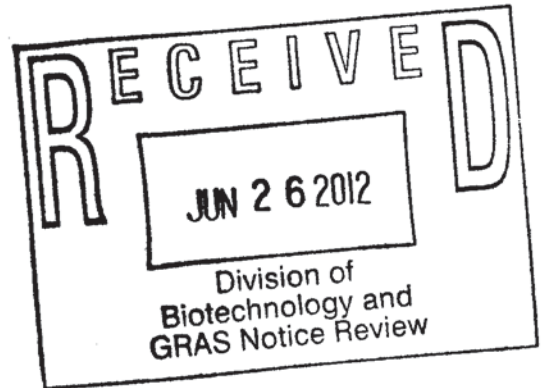




ORIGINAL SUBMISSION

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25 June 2012

Dr. Antonia Mattia
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
U.S. Food & Drug Administration
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740

Reference: Intralytix GRAS Notification for SalmoFresh™

Dr. Mattia:

In accordance with the Federal Register [62 FR 18938 (17 April 1997)] proposed regulations for GRAS notifications (21CFR§170.36), Intralytix is pleased to submit, in triplicate, a notice that the bacteriophage cocktail, SalmoFresh™, has been determined by us to be generally recognized as safe, through scientific procedures, and is exempt from the pre-market approval requirements for the use in foods, generally, to control *Salmonella enterica*.

Also included is a fourth copy for submission to the United States Department of Agriculture's Food Safety and Inspection Service, regarding the use of SalmoFresh™ as a safe and suitable antimicrobial used in the production of poultry products as a processing aid. Some of the efficacy studies included for the FSIS were performed by an industry partner. Therefore, we request that Appendices 1.1 – 1.9 be considered confidential.

If there are any questions or concerns, please contact us.

Sincerely,

(b) (6)

Alexander Sulakvelidze
Vice President
Chief Scientist
Intralytix, Inc.

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Intralix
GRAS Notification:
SalmoFresh™

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COMPUTER TECHNOLOGY SERVICES, INC.

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1 GRAS EXEMPTION CLAIM

Claim of Exemption from the Premarket Approval Requirements Pursuant to Proposed 21CFR§170.36(c)(1)

The bacteriophage cocktail, SalmoFresh™, containing bacteriophages SBA-1781, SKML-39, SPT-1, SSE-121, STML-13-1, and STML-198, has been determined by Intralytix, Inc., to be generally recognized as safe, through scientific procedures, and is exempt from the premarket approval requirements under the intended use conditions described within this notification.

(b) (6)

June 22, 2012

Alexander Sulakvelidze
VP Research and Development
asulakvelidze@intralytix.com

Date

000009

1.1 NAME & ADDRESS OF NOTIFIER

Intralytix, Inc.
701 E Pratt St.
Baltimore, MD 21202
Tel: 877-489-7424
Fax: 410-625-2506

1.2 COMMON OR USUAL NAME

Intralytix produces a *Salmonella*-specific bacteriophage cocktail under the trade name SalmoFresh.

1.3 CONDITIONS OF USE

SalmoFresh™ is intended for use as an antimicrobial to control *Salmonella* on food, generally, when applied to food surfaces up to 1×10^7 PFU/gram of food.

1.4 BASIS FOR THE GRAS DETERMINATION

Pursuant to the GRAS proposed rule, Intralytix has determined that SalmoFresh™ is GRAS through scientific procedures.

1.5 AVAILABILITY OF INFORMATION

The data and information that are the basis for Intralytix's determination of GRAS for SalmoFresh™ are available for review and copying by FDA or will be sent to FDA upon request, made to:

Intralytix
Joelle Woolston
The Columbus Center
701 E Pratt St.
Baltimore, MD 21202
jwoolston@intralytix.com

2 IDENTITY AND SPECIFICATIONS OF SALMOFRESH™

2.1 IDENTITY

SalmoFresh™ consists of a mixture of equal proportions of six individually purified lytic bacteriophages (hereinafter referred to as “Component Phage(s)” or Component Monophage(s).) Each of these monophages is specifically effective against *Salmonella enterica* serotypes. The component phages in SalmoFresh™ were isolated from water of the Inner Harbor of Baltimore and a mix of water samples collected from the Baltimore area.

The current SalmoFresh™ article of commerce is a liquid made up of equal parts of six monophages that combined have a minimum lytic titer of $10.0 \pm 0.33 \log_{10}$ plaque-forming units (PFU) per mL. This article of commerce is a concentrate that is diluted with water at the application site to form the SalmoFresh™ working solution with a maximum mean lytic titer of ca. $9.0 \pm 0.33 \log_{10}$ PFU/mL. It is applied at a rate that ensures the final concentration of phage on the food articles is at or below 1×10^7 PFU/g of food.

2.2 METHOD OF MANUFACTURE

The component monophages of SalmoFresh™ are prepared using an aerobic fermentation process in animal-product free media. For each monophage, its host *Salmonella enterica* strain is grown to a target OD₆₀₀, at which point the culture is infected with the monophage at a previously determined MOI (multiplicity of infection; the ratio of phage to bacteria) and the combination is incubated with aeration and mixing. The suspension is clarified by removal of bacteria by tangential-flow filtration. Following the initial filtration, the monophage is concentrated, washed with 0.1M sodium chloride, then sterilized using 0.22µm filtration. After all six component monophages have each passed quality control specifications, proper volumes of each monophage, and sterile 0.1M sodium chloride as necessary, are combined to form the SalmoFresh™ article of commerce so that:

Each monophage is equally represented

AND

The mean lytic titer is $\geq 10.0 \pm 0.33 \log_{10}$ PFU/mL

The SalmoFresh™ article of commerce is diluted with water at the application site, to form the “working solution” or “working concentration” of SalmoFresh™ with a maximum mean lytic titer of $9.0 \pm 0.33 \log_{10}$ PFU/mL. Figure 1 provides an overall schematic of the process.

2.3 SPECIFICATIONS

Due to the two step manufacturing process, there are two levels of quality control. First, each individual monophage lot is analyzed to ensure it meets the release specifications listed in Table 1 before it can be used to prepare a lot of SalmoFresh™.

Table 1 Product specifications for individual monophage lots

Parameter	Specification	Method
Potency (PFU/mL)	$\geq 10.0 \pm 0.33 \log_{10}$ PFU/mL	Intralytix method M008-03
Bacterial Sterility	No growth	Intralytix method M002-08
Genotypic fingerprinting	Matches reference bands	Intralytix method M002-10

Only after all component monophages have met the release specifications can a lot of SalmoFresh™ be produced. Each lot of SalmoFresh™ is analyzed to ensure it meets the following release specifications listed in Table 2.

Table 2 Product specifications for SalmoFresh™

Parameter	Specification	Method
Potency (PFU/mL)	$\geq 10.0 \pm 0.33 \log_{10}$ PFU/mL	Intralytix method M015-02
Bacterial Sterility	No growth	Intralytix method M002-08
Endotoxin Content (EU/mL)	$\leq 25,000$ EU/mL (at ca. $9.0 \pm 0.33 \log_{10}$ PFU/mL)	Lonza method for LAL QCL-1000
Identity Test	Lyses all reference strains	Intralytix method M015-30

2.4 CHARACTERISTIC PROPERTIES

SalmoFresh™ is a clear to opalescent odorless liquid with a specific gravity of approximately 1.008. The phage component of SalmoFresh™ (maximum working solution at 1×10^9 PFU/mL) is roughly estimated to be 0.0000342% by weight and the remainder is 0.1M sodium chloride. Typical composition of SalmoFresh™ (at the maximum working concentration of ca 1×10^9 PFU/mL) is shown below. The values shown are derived (averages) from the chemical analysis of three separate SalmoFresh™ lots.

Table 3 Typical composition of SalmoFresh™ (maximum working concentration of 1×10^9 PFU/mL)

Property/analysis/composition	SalmoFresh™ Lot# 02111190185	SalmoFresh™ Lot# 02111190210	SalmoFresh™ Lot# 02111190329	SalmoFresh™ typical
Total nitrogen (mg/L)	2.8	2.8	3.1	2.9
pH	7.58	7.71	7.48	7.59
Specific gravity (at 25°C)	1.008	1.008	1.008	1.008
Arsenic (mg/L)	ND	ND	ND	ND
Barium (mg/L)	ND	ND	ND	ND
Cadmium (mg/L)	0.011	0.010	0.011	0.011
Calcium (mg/L)	0.175	0.364	0.230	0.256
Chromium (mg/L)	0.021	0.017	0.032	0.023
Cobalt (mg/L)	ND	ND	ND	ND
Copper (mg/L)	0.071	0.062	0.095	0.076
Iron (mg/L)	0.027	0.183	0.031	0.080
Lead (mg/L)	0.018	0.024	0.024	0.022
Magnesium (mg/L)	ND	ND	ND	ND
Manganese (mg/L)	ND	ND	ND	ND
Nickel (mg/L)	ND	ND	ND	ND
Phosphorus (mg/L)	10.0	10.2	10.2	10.1
Potassium (mg/L)	5.53	5.78	5.19	5.5
Silicon (mg/L)	ND	ND	ND	ND
Sodium (mg/L)	212	230	233	225
Tin (mg/L)	ND	ND	ND	ND
Zinc (mg/L)	0.045	0.056	0.045	0.049
Chloride (mg/L)	341	367	369	359
Nitrate (as N) (mg/L)	0.15	0.15	0.19	0.16
Nitrite (as N) (mg/L)	ND	ND	ND	ND
Total Organic Carbon (mg/L)	21	24	23	23
Total Kjeldahl Nitrogen (mg/L)	2.6	2.6	2.9	2.7
Total Dissolved Solids (mg/L)	580	620	610	603
Total Suspended Solids (mg/L)	ND	ND	ND	ND
Total Phosphorous (mg/L)	11.0	11.8	11.5	11.4
Silica (mg/L)	2.25	2.27	2.75	2.4
Endotoxin content (EU/mL)	4559	3872	2933	3788

ND = none detected

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2.5 PHAGE CLASSIFICATION

The component phages in SalmoFresh™ were isolated by Intralytix's scientists from the environment. Each monophage was fully characterized by a variety of methods, including pulse-field gel electrophoresis (PFGE,) restriction fragment length polymorphism (RFLP,) electron microscopy (EM,) full-genome sequence analysis, lytic activity against *Salmonella enterica* strains, and lytic activity against non-*Salmonella enterica* strains.

Name: SBA-1781
 Order: Caudovirales
 Family: *Myoviridae*
 Properties: Double-stranded DNA, Lytic

Name: SKML-39
 Order: Caudovirales
 Family: *Myoviridae*
 Properties: Double-stranded DNA, Lytic

Name: SPT-1
 Order: Caudovirales
 Family: *Myoviridae*
 Properties: Double-stranded DNA, Lytic

Name: SSE-121
 Order: Caudovirales
 Family: *Myoviridae*
 Properties: Double-stranded DNA, Lytic

Name: STML-13-1
 Order: Caudovirales
 Family: *Myoviridae*
 Properties: Double-stranded DNA, Lytic

Name: STML-198
 Order: Caudovirales
 Family: *Myoviridae*
 Properties: Double-stranded DNA, Lytic

2.6 POTENTIAL HUMAN TOXICANTS

The *Salmonella enterica* host strains are Gram-negative bacteria. As with all Gram-negative bacteria, they produce bacterial endotoxin or lipopolysaccharide (LPS.) Intralytix tests every lot of SalmoFresh™ for LPS to ensure it meets the release criteria. Endotoxins are further discussed below, in Sections 4.1.3, 4.2.1.3, and 4.2.2.2.

2.7 STABILITY

The proposed shelf life of SalmoFresh™ article of commerce is one year when stored at 2–6°C in a dark, UV-protected area.

3 SELF-LIMITING LEVELS OF USE

The proposed use for SalmoFresh™ is as an antimicrobial processing aid for foods that are at high risk to be contaminated with *Salmonella enterica*. The purpose of SalmoFresh™ is to significantly reduce or eliminate *Salmonella enterica* in the final product.

The self-limiting levels of use are:

- Due to the cost of the product, the end-user would use the minimum dose required to achieve a significant reduction or elimination of *Salmonella enterica*.
- Once the *Salmonella enterica* contamination is depleted, the phage will slowly decrease in number due to a lack of host.
- Phages are susceptible to many environmental factors, including sunlight, heat, and UV light. Exposure to these should cause the number of phage to decrease.

4 BASIS OF DETERMINATION GRAS: GRAS THROUGH SCIENTIFIC PROCEDURES

4.1 COMPONENTS OF SALMOFRESH™

SalmoFresh™ is a mixture of component bacteriophages together with added sodium chloride and small amounts of residual production by-products. The primary active ingredient is not a single chemical substance but a mixture of naturally-occurring bacteriophages. In the appropriate sections below, we consider separately the safety of the:

Phages (active component)

Added salts

Manufacturing by-products

4.1.1 Monophages

The safety and ubiquity of bacteriophages have been well established. The pertinent safety data on bacteriophages is reviewed below. The published literature on phages, and other information developed by Intralytix, shows that:

- Bacteriophages are the most ubiquitous organisms on earth. For example, one milliliter of non-polluted stream water has been reported (Bergh et al., 1989) to contain approximately 2×10^8 PFU of phages/mL, and the total number of phages on this planet has been estimated to be in the range of $10^{30} - 10^{32}$ (see <http://www.asm.org/division/m/M.html> and Brussow & Hendrix, 2002.) This abundance of phages in the environment, and the continuous exposure of animals to them, explains the extremely good tolerance of mammalian organisms to phages.
- Phages have been used therapeutically in humans for more than 80 years, without any serious side effects. During the long history of using phages as therapeutic agents in Eastern Europe and the former Soviet Union (and, before the antibiotic era, in the United States, France, Australia, and other countries), phages have been administered to humans:
 - orally, in tablet or liquid formulations,
 - rectally,
 - locally (skin, eye, ear, nasal mucosa, etc.); in tampons, rinses and creams,
 - as aerosols or intrapleural injections, and
 - intravenously

There have been virtually no reports of serious complications associated with their use. Recent reviews summarize the results of some of the human therapy studies involving bacteriophages (Alisky et al., 1998; Sulakvelidze et al., 2001; Summers, 2001.)

- Phages have also been administered to humans for non-therapeutic purposes without any recorded illness or death. To give just a few examples, phage preparations have been used extensively to monitor humoral immune function in humans in the United States in the 1970s-1990s, including in patients with Down's syndrome, the Wiskott-Aldrich syndrome and immunodeficient patients (Lopez et al., 1975; Ochs et al., 1992; Ochs et al., 1982; Ochs et al., 1993a.) In some of the studies (including several studies performed by the FDA), the purified phages were injected intravenously into HIV-infected patients or other immunodeficient individuals without any apparent side effects (Fogelman et al., 2000; Ochs et al., 1971; Ochs et al., 1993b.)
- Phages have also been administered to humans via various sera and FDA-approved vaccines commercially available in the United States (Merril et al., 1972; Milch & Fornosi, 1975; Moody et al., 1975.)
- The biology of phages has been exhaustively studied. These studies have clearly shown that phages are obligate intracellular parasites of bacteria and are not infectious in humans or other mammals.

- Bacteriophages are common commensals of the human gut, and they are likely to play an important role in regulating the diversity and population structure of various bacteria in human GI tracts. For example, phages capable of infecting *E. coli*, *Bacteroides fragilis* and various *Salmonella* serotypes have been isolated from human fecal specimens in concentrations as high as 10^5 PFU/100 g of feces (Calci et al., 1998; Furuse et al., 1983; Havelaar et al., 1986.) The recent data based on metagenomic analyses (using partial shotgun sequencing) of an uncultured viral community from human feces suggested that bacteriophages are the second most abundant category after bacteria in the uncultured fecal library (Breitbart et al., 2003.)
- No adverse immunologic or allergic sequelae have ever been reported because of human or animal exposure to phages (Alisky et al., 1998; Sulakvelidze et al., 2001.)
- Bacteriophages are commonly consumed via drinking water (Armon et al., 1997; Armon & Kott, 1993; Grabow & Coubrough, 1986; Lucena et al., 1995.)
- Bacteriophages are commonly consumed via various foods. In this context, bacteriophages have been commonly isolated from a wide range of food products, including ground beef, pork sausage, chicken, farmed freshwater fish, common carp and marine fish, oil sardine, raw skim milk, and cheese (Atterbury et al., 2003; Gautier et al., 1995; Greer, 2005; Hsu et al., 2002; Kennedy et al., 1986; Kennedy et al., 1984; Whitman & Marshall, 1971.) Several studies have suggested that 100% of the ground beef and chicken meat sold at retail contain various levels of various bacteriophages. To give just a few examples, bacteriophages were recovered from 100% of examined fresh chicken and pork sausage samples and from 33% of delicatessen meat samples analyzed by Kennedy et al (1984.) The levels ranged from 3.3 to 4.4×10^{10} PFU/100 g of fresh chicken, up to 3.5×10^{10} PFU/100 g of fresh pork, and up to 2.7×10^{10} PFU/100 g of roast turkey breast samples. In another study (Kennedy et al., 1986), samples of fresh chicken breasts, fresh ground beef, fresh pork sausage, canned corned beef, and frozen mixed vegetables were examined for the presence of coliphages. Although only three ATCC strains of *E. coli* were used as indicator host strains, coliphages were found in 48 to 100% of the various food samples examined.
- Because of the highly specific nature of their lytic cycle, and because of the extremely common exposure of humans and animals to bacteriophages (including daily consumption of bacteriophages with various foods and drinking water), bacteriophages do not deleteriously affect the GI microflora. For example, when *E. coli*-specific phage T4 was administered orally to 15 healthy adult volunteers, it did not cause a decrease in total fecal *E. coli* counts. In addition, no substantial phage T4 replication on the commensal *E. coli* population was identified, and no adverse events related to phage application were observed in any of the volunteers (Bruttin & Brussow, 2005.)
- Bacteriophages are commonly consumed by animals (including agriculturally-important species) via various foods. For example, in a recent study from Texas A&M University (Maciorowski et al., 2001), male-specific and somatic coliphages were detected in all animal feeds, feed ingredients, and poultry diets examined, even after the samples were stored at -20°C for 14 months.

4.1.1.1 Lytic phages are GRAS

All lytic phages are, by nature, GRAS. There are two major types of phages: “virulent” (also called “lytic”) and “temperate” (often mistakenly called “lysogenic”). Lytic phages lyse host bacteria without integrating into the host genome. In contrast, temperate phages may integrate into the host genome and a small subset of these may theoretically transduce undesirable bacterial genes, such as those encoding toxins or antibiotic resistance. Both lytic and temperate phages are extremely common in the environment, the human and animal gut, the human oral cavity, foods sold at retail, sewage, and many other places that we encounter daily. Humans shed large numbers of both lytic and temperate phages into the environment every day – estimated to be on the order of 4×10^9 single phage daily per person. (Sulakvelidze & Barrow, 2005) Temperate phages are found in almost all bacterial genera, including *Staphylococcus*, *Vibrio*, *Pseudomonas*, *Salmonella*, *Shigella*, *Bacillus*, *Corynebacterium*, *Listeria*, and *Streptococcus* (Jacob & Wollman, 1959; Schicklmaier & Schmieger, 1995; Eggers et al., 2001; Langley et al., 2003.) Indeed, some strains can release as many as five different types of temperate phages. Although the possibility of added gene transfer events is highly unlikely to bring danger to any individual consuming temperate phages, the use of such phages on an industrial scale could increase the overall risk of potentially harmful genes being acquired by new bacterial strains. Therefore, Intralytix identifies and uses only lytic phages in its phage preparations (including SalmoFresh™).

4.1.1.2 SalmoFresh™ monophages are GRAS

The component phages in SalmoFresh™ were isolated by Intralytix’s scientists from both water of the Inner Harbor of Baltimore and a mix of water samples collected from the Baltimore area. Each was characterized by various approaches, including electron microscopy, genotypic fingerprinting, and full genome sequence analysis. The component phages in SalmoFresh™ are members of the *Myoviridae* double-stranded DNA phage family, as defined by the International Committee on the Taxonomy of Viruses (ICTV) and by Ackermann and Berthiaume, 1995.

Intralytix has fully sequenced all component monophages included in SalmoFresh™. This approach is used to exclude bacteriophages carrying sequences encoding undesirable genes, and phages displaying prior evidence of transduction.

Intralytix excludes all bacteriophages carrying sequences encoding any undesirable genes. Undesirable genes include genes encoding bacterial toxins (including genes listed in 40 CFR § 725.421) or genes associated with drug resistance. Undesirable genes are identified by comparing a complete bacteriophage sequence to all sequences contained in GenBank and other databases available through the National Center for Biotechnology Information website of the National Library of Medicine using the BLASTn program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

The cut-off *e*-value level for the latter analysis was 1×10^{-4} , which detects virtually all undesirable genes in the phages’ genomes. In practice, significant matches are considered to be those with *e*-values of $\leq 10^{-5}$ (Miller et al., 2003.) Therefore, our proposed cut-off value provides a very strong (10-fold higher than the proposed 10^{-5} cut-off) assurance that undesirable genes are not missed during the analysis.

Intralytix has sequenced the complete genome of each phage incorporated into SalmoFresh™. Table 4 summarizes their genome properties. Analysis of the sequences yielded the following results:

No toxin genes have been identified among the open reading frames of the annotated genomes of any of the six monophages.

No 16S ribosomal RNA genes have been identified among annotated genomes of any of the six monophages.

No antibiotic resistance genes have been identified among annotated genomes of any of the six monophages.

Summary: The approach of obtaining the full nucleotide sequence for each commercialized phage and complete bioinformatics analysis of all open reading frames insures that no detrimental genes are present in any of the phages used. This provides the fullest assurance of the phage safety as can presently be obtained by any method.

Table 4 Genome size and composition of phages contained in SalmoFresh™

Phage	ATCC #	GenBank Accession #	GC%	Size (bp)	Number of Open Reading Frames (ORFs)	Undesirable genes
SBA-1781	PTA-5282	JX181814 - JX181821	39	88 124	741	None
SKML-39	PTA-12380	JX181829	50	159 624	1547	None
SPT-1	PTA-5281	JX181822 - JX181823	39	87 248	725	None
SSE-121	PTA-5283	JX181824	45	147 745	1455	None
STML-13-1	PTA-8365	JX181826 - JX181828	51	161 646	1650	None
STML-198	PTA-12381	JX181825	37	158 160	1350	None

4.1.1.3 SalmoFresh™ is specific to *Salmonella*

Lytic activity of SalmoFresh™ is targeted against *Salmonella enterica* strains. SalmoFresh™ has been screened for its lytic activity against over 900 *Salmonella* strains in the Intralytix collection, representing more than 50 serotypes. As shown in Table 5, SalmoFresh™ is very effective against several serotypes.

Table 5 A selection of *Salmonella* serotypes from Intralytix's collection and the percent kill of each by SalmoFresh™ at 1x10⁹ PFU/mL.

Serotype*	# strains in Intralytix collection	Percent kill (1x10 ⁹ PFU/mL SalmoFresh™)
Typhimurium	182	100%
Enteritidis	136	100%
Hadar	115	99%
Heidelberg	40	100%
Newport	27	100%
Kentucky	25	100%
Georgia	19	89%
Agona	11	100%
Grampian	8	100%
Senftenberg	8	100%
Alachua	5	80%
Infantis	5	100%
Reading	5	100%
Schwarzengrund	5	80%
Thompson	5	100%
All strains	916	95%

* Serotypes listed are those represented by ≥5 strains in Intralytix's collection.

SalmoFresh™ is also highly specific. Table 6 shows that SalmoFresh™ does not lyse any of the non-*Salmonella enterica* gram positive strains examined. These strains include 5 strains each of *Staphylococcus aureus* and *Listeria species*. SalmoFresh™ also does not lyse several non-*Salmonella* gram negative strains, including 5 strains each of *Acinetobacter baumannii*, various *Enterococcus species*, *Pseudomonas aeruginosa*, and various *Shigella species*. Of the 5 strains of *E. coli* tested, SalmoFresh™ was able to lyse a few. This species is closely related to *Salmonella*, also being a member of the Enterobacteriaceae family. Bruttin & Brussow (2005) demonstrated orally administration of *E. coli*-specific phage T4 did not affect fecal *E. coli* counts and had no adverse effects in any volunteers. Therefore, SalmoFresh™ would also have no deleterious affect upon the natural gut flora.

Table 6 Lytic activity of SalmoFresh™ against non-*Salmonella enterica* strains of bacteria

Non-Salmonella enterica strains		Species	Susceptibility to SalmoFresh™ (1x10 ⁹ PFU/mL)
Intralytix ID	Original ID		
Sa36	ATCC25923	<i>Staphylococcus aureus</i>	-
Sa37	ATCC29213	<i>Staphylococcus aureus</i>	-
Sa211	ATCC700699	<i>Staphylococcus aureus</i>	-
Sa298	ATCC49775	<i>Staphylococcus aureus</i>	-
Sa299	ATCC14458	<i>Staphylococcus aureus</i>	-
Lm 314	ATCC19117	<i>Listeria monocytogenes</i>	-
Lm 315	ATCC19118	<i>Listeria monocytogenes</i>	-
L.innocua 316	ATCC51724	<i>Listeria innocua</i>	-
Lm 317	ATCC19116	<i>Listeria monocytogenes</i>	-
L.innocua 318	ATCC33090	<i>Listeria innocua</i>	-
Ab3	ATCC19606	<i>Acinetobacter baumannii</i>	-
Ab4	HER1401	<i>Acinetobacter baumannii</i>	-
Ab5	4308-2	<i>Acinetobacter baumannii</i>	-
Ab6	3247-1	<i>Acinetobacter baumannii</i>	-
Ab7	1673-2	<i>Acinetobacter baumannii</i>	-
E102	WCC188	<i>Enterococcus spp.</i>	-
E402	ATCC11823	<i>Enterococcus faecalis</i>	-
E403	ATCC19433	<i>Enterococcus faecalis</i>	-
E404	1133455	<i>Enterococcus avium</i>	-
E405	1126611	<i>Enterococcus faecalis</i>	-
Pa76	ATCC10145	<i>Pseudomonas aeruginosa</i>	-
Pa161	ATCC15692	<i>Pseudomonas aeruginosa</i>	-
Pa162	ATCC51674	<i>Pseudomonas aeruginosa</i>	-
Pa163	ATCC43390	<i>Pseudomonas aeruginosa</i>	-
Pa164	ATCC39324	<i>Pseudomonas aeruginosa</i>	-
SH.d1	514	<i>Shigella dysenteriae</i>	-
SH.f6	045-311082	<i>Shigella flexneri</i>	-
SH.f20	300	<i>Shigella flexneri</i> 2	-
SH.s43	90	<i>Shigella sonnei</i>	-
SH.s52	ATCC9290	<i>Shigella sonnei</i>	-
Ec147	ATCC43895	<i>Escherichia coli</i> O157:H7	+
Ec148	ATCC35401	<i>Escherichia coli</i> O78:H11	-
Ec150	ATCC700728	<i>Escherichia coli</i> O157:H7	+
Ec154	ATCC11303	<i>Escherichia coli</i>	-
Ec155	ATCC12435	<i>Escherichia coli</i>	-

+ Lysed by phage cocktail

- Not lysed by phage cocktail

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4.1.2 Sodium chloride

Sodium chloride “salt” is the prototype in 21 CFR § 182.1 (a) of an ingredient that is so obviously GRAS that FDA has not listed it as GRAS.

4.1.3 By-products

The *Salmonella enterica* host strains are Gram-negative bacteria. As with all Gram-negative bacteria, they produce bacterial endotoxin or LPS. Intralytix tests every lot of SalmoFresh™ to ensure its LPS levels fall below the stringent release criteria. Endotoxins are further discussed below, in Sections 4.2.1.3 and 4.2.2.2. Otherwise, there are no known potentially noxious by-products in SalmoFresh™.

4.2 MANUFACTURING OF SALMOFRESH™

SalmoFresh™ is prepared by cultivation of individual host *Salmonella enterica* strain/phage combinations followed by filtration, concentration, wash, and final sterile filtration. After each monophage passes quality control, the monophages are combined with 0.1M sodium chloride to form the SalmoFresh™ concentrate. Final filtration is then carried out with a sterilizing grade filter.

4.2.1 Starting components

There are four starting components for manufacture of SalmoFresh™ components monophages:

- Animal-product free media
- Antifoam
- Host strains
- Monophages

The safety of each is considered separately below.

4.2.1.1 Animal-product free media

The animal-product free media is a vegan custom blend. The main components are described here and have an existing regulatory status as regulated GRAS ingredients or additives.

Phytone Peptone and *Soytone*: Peptones are GRAS affirmed at 21 CFR § 184.1553 for use as processing aids, among other uses, at levels not to exceed good manufacturing practice. Peptones are protein hydrolysates consisting of free amino acids and short peptides in an aqueous salt solution.

Yeast Extract: Yeast extract is a commonly used food ingredient. For example, baker's yeast extract is GRAS affirmed as a flavoring agent or adjuvant at up to 5% in foods generally. 21 CFR § 184.1983.

Sodium Chloride: Sodium chloride “salt” is the prototype in 21 CFR § 182.1 (a) of an ingredient that is so obviously GRAS that FDA has not listed it as GRAS.

Magnesium Sulfate: Magnesium sulfate salt is GRAS affirmed at 21 CFR § 184.1443 for use as a processing aid, among other uses, at levels not to exceed good manufacturing practice. Magnesium sulfate is a component of the animal-product free growth media used in SalmoFresh™ production.

4.2.1.2 Antifoaming agent

P2000 antifoam is polypropylene glycol-based, Kosher-certified product, approved for a variety of food additive uses, both direct and indirect (The Dow Chemical Company, Midland, Michigan; <http://www.dow.com>) Antifoam is used in the initial fermentation of the individual monophages.

4.2.1.3 Host strains

The component monophages are produced on several *Salmonella enterica* serotypes. Strains from Intralytix’s collection of *Salmonella enterica* strains were selected as the production hosts for SalmoFresh™ component monophages (Table 7). The *Salmonella enterica* host strains were characterized at Intralytix for their biochemical properties, background genomic composition/type, the presence of endogenous phage(s), and their susceptibility to five commonly prescribed antibiotics (cefotaxime, ceftriaxone, ciprofloxacin, levofloxacin, and sulfamethoxazole/trimethoprim.)

Table 7 Summary of *Salmonella enterica* host strain specifications

Phage	Current <i>S. enterica</i> host strain	Serotype	Biochemistry	PFGE	Endogenous phage	Antibiotic susceptibility
SBA-1781	S.H178	Hadar	<i>Salmonella</i> spp	+	-	Susceptible to all tested
SKML-39	S.K39	Kentucky	<i>Salmonella</i> spp	+	-	Susceptible to all tested
SPT-1	S.E378	Enteritidis	<i>Salmonella</i> spp	+	-	Susceptible to all tested
SSE-121	S.A121	Agona	<i>Salmonella</i> spp	+	-	Susceptible to all tested
STML-13-1	S.E236	Enteritidis	<i>Salmonella</i> spp	+	-	Susceptible to all tested
STML-198	S.T198	Typhimurium	<i>Salmonella</i> spp	+	-	Susceptible to all tested

The *Salmonella enterica* host strains are not known to produce any enterotoxins that could compromise the safety of the final product. Therefore, the only production host strain-related toxin that is relevant for SalmoFresh™ safety is endotoxin or LPS. As with all Gram-negative bacteria, *Salmonella* outer membrane contains lipopolysaccharide or LPS (Wang & Quinn,

2010). If sufficiently high amounts of LPS enter human bloodstream, it can trigger the signaling cascade for macrophage/endothelial cells to secrete pro-inflammatory cytokines and nitric oxide that may lead to "endotoxic shock." Because SalmoFresh™ phages are propagated in *Salmonella* host strains, host cells lyse during the process (as the result of phage lytic cycle) and *Salmonella* LPS is present in the resulting phage lysates. Most of the endotoxin is expected to be removed during phage purification process. However, as a standard quality control protocol, Intralytix analyzes every SalmoFresh™ batch for the presence and levels of the LPS endotoxin in the final product. All product lots must be at or below 250,000 endotoxin unit (EU)/mL at 1×10^{10} PFU/mL level in order to pass the release criteria for LPS. This level is very safe, and is based upon the levels of endotoxins that are found naturally in healthy human saliva (Leenstra et al., 1996). See Section 4.3.2.3 for discussion of dietary intake.

4.2.1.4 Monophages

The safety of monophages is discussed in Section 4.1.1.2.

4.2.2 Quality Control

4.2.2.1 Monophages

The following tests are performed upon each monophage lot:

Potency test

The potency test measures the lytic titer of each monophage lot, by determining the number of plaque forming units per milliliter (PFU/mL.) The specification for each monophage lot is a titer of $\geq 10.0 \pm 0.33 \log_{10}$ PFU/mL. Lots failing to meet the specification due to a low titer may be appropriately concentrated and retested.

Bacterial sterility

Bacterial sterility is a determination of the viable microbial contamination in a phage solution. Briefly, samples of each monophage solution are tested by a) direct plating onto non-selective agar and b) after enrichment. The specification is that each monophage lot must be bacteriologically sterile. Lots failing the sterility test will be re-filtered and retested. Lots repeatedly failing to meet the specification will be discarded.

Genotypic fingerprinting

Genotypic fingerprinting, through restriction fragment length polymorphism (RFLP,) is used to confirm the identity of each monophage lot. The specification for RFLP is that all major bands in the reference pattern must be present. Lots failing the RFLP test will be discarded.

4.2.2.2 SalmoFresh™

The following tests are performed upon each batch of SalmoFresh™:

Potency test

The potency test method is based on determining the mean titer (PFU/mL) of the SalmoFresh™ preparation. The specification for this test is SalmoFresh™ has a mean titer of $\geq 10.0 \pm 0.33 \log_{10}$ PFU/mL. Lots failing to meet the specification due to a low titer may be appropriately concentrated and retested.

Bacterial sterility

Bacterial sterility is a determination of the viable microbial contamination in a phage solution. Briefly, samples of each batch of SalmoFresh™ are tested by a) direct plating onto non-selective agar and b) after enrichment. The specification for this test is that SalmoFresh™ must be bacteriologically sterile. Lots failing the sterility test will be re-filtered and retested. Lots repeatedly failing to meet the specification will be discarded.

Endotoxin content test

Endotoxins are toxins associated with host bacteria that are removed from phage preparations. Commercial kits specifically for measurement of this endotoxin are used by Intralalytix. The specification for this test is each lots of SalmoFresh™ must contain $\leq 25,000$ EU/mL in SalmoFresh™ (at maximum working concentration ca. $9.0 \pm 0.33 \log_{10}$ PFU/mL) Lots failing to meet the specification can be washed with sterile 0.1M saline and retested.

Identity test

The identity test verifies that all phages claimed to be present in SalmoFresh™ are actually present. Briefly, six *Salmonella enterica* strains, each of which is susceptible to only one component monophage, are screened for lysis by SalmoFresh™. The specification for this test is that all reference bacterial strains are lysed by the cocktail. Lots that fail to meet the specification may be retested. Lots repeatedly failing the specification may be supplemented with the missing component monophage and retested.

4.3 APPLICATION RATES AND DIETARY INTAKE

4.3.1 Application rates

The current SalmoFresh™ article of commerce is a 10X concentrate that must be diluted with water at the application site to form the SalmoFresh™ working solution with a mean lytic titer of ca. $9.0 \pm 0.33 \log_{10}$ PFU/mL. It is applied at a rate that ensures the final concentration of phage on the food articles is at or below 1×10^7 PFU/g of food. Future preparations may be sold in more concentrated form, but the accompanying instructions for dilution and application rate will be appropriately adjusted to ensure the final concentration of phage on the food articles is always at or below ca. 1×10^7 PFU/g of food.

4.3.2 Dietary intakes

According to the CDC (2011), in 2008, poultry was the food responsible for the most Salmonella outbreaks that could be attributed to a single food commodity. Because poultry is the food most likely to be at risk, the following calculations are based upon the average American's intake of poultry and assume the maximum scenario, that 100% of poultry produced in the US is SalmoFresh™ treated.

To determine the daily intake of all turkey and chicken products in all forms for the US population as a whole, the American Meat Institute estimates of per capita intake data were used. These estimates are based on the weight of poultry sold in retail stores. In the year 2009, each American consumed approximately 81 lbs of chicken and 16.9 lbs of turkey, for a total of about 98 lbs of poultry per year per person (AMI fact sheet.)

4.3.2.1 Dietary intakes for SalmoFresh™

The following calculation to determine the maximum (worst-case scenario) consumption of SalmoFresh™ by the average American uses the highest rate of SalmoFresh™ application (1×10^7 PFU/g):

Weight of poultry consumed per day per person:

$$\frac{98 \text{ lbs poultry}}{\text{year}} \times \frac{\text{year}}{365 \text{ day}} \times \frac{1000 \text{ g}}{2.2 \text{ lb}} = \frac{122 \text{ g poultry}}{\text{day}}$$

Volume of SalmoFresh™ applied per gram of poultry:

$$\frac{1 \times 10^7 \text{ PFU}}{\text{g poultry}} \times \frac{1 \text{ mL SalmoFresh™}}{1 \times 10^9 \text{ PFU}} = \frac{0.01 \text{ mL SalmoFresh™}}{\text{g poultry}}$$

Volume of SalmoFresh™ consumed per day per person:

$$\frac{122 \text{ g poultry}}{\text{day}} \times \frac{0.01 \text{ mL SalmoFresh™}}{\text{g poultry}} = \frac{1.22 \text{ mL SalmoFresh™}}{\text{day}}$$

The volume of SalmoFresh consumed per day due to poultry would be about 1.22mL or the equivalent of a ¼ teaspoon. This volume is negligible and safe.

4.3.2.2 Dietary intakes for SalmoFresh™ phages

The following calculation determines the approximate weight of phages consumed per day, again assuming the maximum rate (1×10^7 PFU/g) of SalmoFresh™ application:

Total phages (PFU) consumed per day:

$$\frac{1.22 \text{ mL SalmoFresh}^{\text{TM}}}{\text{day}} \times \frac{1 \times 10^9 \text{ PFU}}{\text{mL}} = \frac{1.22 \times 10^9 \text{ PFU}}{\text{day}}$$

Weight of total phages consumed/day (in micrograms):

$$\frac{1.22 \times 10^9 \text{ PFU}}{\text{day}} \times \frac{3.45 \times 10^{-16} \text{ g}}{\text{phage}} \times \frac{1 \times 10^6 \mu\text{g}}{\text{g}} = \frac{0.421 \mu\text{g}}{\text{day}}$$

Where:

$$3.45 \times 10^{-16} \text{ g} = \text{mass of one phage}$$

Assuming the average diet is 3 kg/day, the dietary concentration of phages is:

$$\frac{0.421 \mu\text{g}}{\text{day}} \times \frac{\text{day}}{3 \text{ kg}} = 0.140 \text{ ppb}$$

The weight of phages consumed per day via SalmoFreshTM would be 0.421 μg, or 0.140 ppb in a 3 kg diet. This is insignificant.

4.3.2.3 Dietary intake of endotoxin

Normal saliva contains approximately 1 mg endotoxin per mL (Leenstra et al., 1996.) For endotoxin, 1 EU/mL is approximately equal to 1 ng/mL. This means that the 1 mg/mL of endotoxin in saliva is equivalent to approximately 1×10^6 EU/mL. Specification for SalmoFreshTM lots for endotoxin is $\leq 25,000$ EU/mL at 1×10^9 PFU/mL.

The approximate daily volume of SalmoFreshTM consumed is 1.22 mL (see Section 4.3.2.1.) Again using the worst case scenario (maximum allowable endotoxin level by specification), the maximum amount of endotoxin consumed via SalmoFreshTM is thus:

$$\frac{1.22 \text{ mL SalmoFresh}^{\text{TM}}}{\text{day}} \times \frac{2.5 \times 10^4 \text{ EU}}{\text{mL SalmoFresh}^{\text{TM}}} = \frac{3.05 \times 10^4 \text{ EU}}{\text{day}}$$

Humans produce approximately 500 to 750 mL of saliva per day. Using the lower, more conservative number, healthy humans consume from saliva:

$$\frac{500 \text{ mL saliva}}{\text{day}} \times \frac{1 \times 10^6 \text{ EU}}{\text{mL saliva}} = \frac{5 \times 10^8 \text{ EU}}{\text{day}}$$

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The maximal amount contributed by SalmoFresh™ would thus constitute 0.0061% of the daily load of endotoxin from saliva. The level of endotoxin found in SalmoFresh™ is therefore considered safe.

4.3.2.4 Sodium and potassium content

From Section 2.4, the highest value obtained for sodium content in a SalmoFresh™ lot was 233 mg/L. From this value, the amount of sodium contributed to the daily diet via SalmoFresh™ on poultry can be calculated as follows:

$$\frac{233 \text{ mg sodium}}{1000 \text{ mL SalmoFresh™}} \times \frac{1.22 \text{ mL SalmoFresh™}}{\text{day}} = \frac{0.284 \text{ mg sodium}}{\text{day}}$$

The amount of sodium per serving contributed by SalmoFresh™, 0.284 mg, is below the level that would change nutritional content labeling by the end-user. The recommended daily allowance of sodium is 2,400 mg (21 CFR § 101.9(c)(9)). The amount of sodium per day contributed by SalmoFresh™ thus represents 0.012% of the RDA and is negligible.

From Section 2.4, the highest value obtained for potassium content in a SalmoFresh™ lot was 5.78 mg/L. From this value, the amount of potassium contributed to the daily diet via SalmoFresh™ on poultry can be calculated as follows:

$$\frac{5.78 \text{ mg potassium}}{1000 \text{ mL SalmoFresh™}} \times \frac{1.22 \text{ mL SalmoFresh™}}{\text{day}} = \frac{0.007 \text{ mg potassium}}{\text{Day}}$$

The amount of potassium per serving contributed by SalmoFresh™, 0.007 mg, is well below the level that would change nutritional content labeling by the end-user. The recommended daily allowance of potassium is 3,500 mg (21 CFR § 101.9(c)(9)). The amount of potassium per serving contributed by SalmoFresh™ thus represents 0.0002% of the RDA and is negligible.

4.4 SUBSTANTIAL EQUIVALENCE TO PREVIOUSLY APPROVED PRODUCTS

4.4.1 Previously approved bacteriophage cocktails

Several lytic bacteriophage products targeting various bacterial pathogens have already been designated GRAS and/or cleared for food safety usage and other applications by a number of regulatory agencies:

- ListShield™ (formerly known as LMP-102,) a phage preparation containing six lytic *Listeria monocytogenes*-specific phages, is FDA-cleared as a food additive (21 CFR §172.785.)

- ListShield™ is also listed by the FSIS for use on various RTE meats and poultry products (FSIS Directive 7120.1.)
- ListShield™ is also EPA-registered for use on non-food surfaces in food processing plants to prevent or significantly reduce contamination of *Listeria monocytogenes* (EPA registration #74234-1.)
- Listex™, a phage preparation containing a single *Listeria monocytogenes* lytic phage, P100, is GRAS (GRAS Notice No. 000218.)
- Listex™ is also listed by the FSIS for use as processing aid when applied at a level of 1×10^7 to 1×10^9 PFU/g food product (FSIS Directive 7120.1.)
- EcoShield™ (formerly ECP-100™,) a phage preparation containing three lytic *E. coli* O157:H7-specific phages, is FDA-cleared for use as a food contact substance (FCN No. 1018.)
- EcoShield™ is also listed by the FSIS for use as processing aid on red meat parts and trim prior to grinding (FSIS Directive 7120.1.)
- AgriPhage™, a phage preparation targeting *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *Tomato*, is EPA-registered for use on tomatoes and peppers (EPA Reg. No. 67986-1.)
- Two bacteriophage preparations – one *Salmonella* targeting and one *E. coli* O157:H7 targeting – are listed by the FSIS for use as processing aids on the hides and feathers of live animals before slaughter (FSIS Directive 7120.1.)

Several regulatory agencies are represented in the preceding list, each of which separately determined that a different bacteriophage preparation was safe and effective. The variety of these previously cleared or registered bacteriophage preparations attests to the general safety of bacteriophages and therefore supports their natural GRAS status. SalmoFresh™ is substantially equivalent to the above bacteriophage cocktails and therefore is also GRAS.

4.5 SUMMARY AND BASIS FOR GRAS

SalmoFresh™ is an all-natural product made of six *Salmonella*-specific lytic bacteriophages from the *Myoviridae* family. All phages included in SalmoFresh™ are lytic phages that were obtained from the environment in the USA and have not been genetically manipulated in any way. The component phages of SalmoFresh™ have been rigorously characterized (including full genome sequencing).

Phages are omnipresent in the environment. Bacteriophages are the oldest, most ubiquitous organisms on earth, with their numbers on Earth estimated to be between 10^{30} and 10^{32} . Phages are present everywhere – including in our mouths, on our skin, and within our gastrointestinal tracks. They are also common and natural ingredients of all fresh, unprocessed foods. The omnipresence of phages (including in foods) and their daily consumption by humans makes them naturally GRAS.

In further recognition of their safety, several lytic bacteriophage products targeting various bacterial pathogens have already been designated GRAS and/or cleared for food safety usage and other applications by a number of regulatory agencies.

Although all lytic bacteriophages are, by nature, GRAS, the phages in SalmoFresh™ must be verified to be lytic and to not contain any undesirable genes listed in 40 CFR § 725.421. All monophages included in SalmoFresh™ belong to the *Myoviridae* family of double-stranded DNA bacteriophages. Many *Myoviridae* phages are known to be excellent lytic phages and are increasingly being used in various phage cocktails, including several previously FDA and EPA cleared bacteriophage preparations.

The genomes of the six bacteriophages in SalmoFresh™ have been sequenced. Bioinformatic analysis of the component phages' sequences shows none contain any undesirable genes listed in 40 CFR §725.421. Furthermore, no antibiotic resistance gene, no 16S RNA sequences, or other known toxin genes were identified in any of the phage genomes.

SalmoFresh™ is manufactured and QC-tested using Intralytix's standard procedures. These procedures have been reviewed by the FDA for manufacturing of Intralytix's bacteriophage food safety products, ListShield™ (21 CFR §172.785) and EcoShield™ (FCN No. 1018,) and are currently used to manufacture commercial lots of these products.

The only manufacturing byproduct of potential concern is LPS. Intralytix tests every lot of SalmoFresh™ for LPS to ensure it meets the release criteria. The LPS levels of the SalmoFresh™ (at maximum working concentration ca. 1×10^9 PFU/mL) must be below 25,000 EU/mL for the lot to be released. This standard is the same as the maximum LPS level previously cleared by the FDA for EcoShield™ (per FCN 1018.) Typical LPS levels in SalmoFresh™ are 3,788 EU/mL, which is more than 6 times less than the allowed LPS levels for EcoShield™.

SalmoFresh™ is produced on animal-product free media. The final SalmoFresh™ product contains no preservatives, known allergenic substances, or additives. SalmoFresh™ has been certified both Kosher and Halal. SalmoFresh™ is also eligible for OMRI-listing, which will be pursued dependent upon market demands.

The proposed application rate for SalmoFresh™ is up to 1×10^7 PFU SalmoFresh™ per gram of food article. Assuming the maximum application rate of 1×10^7 PFU/g of poultry, the average daily consumption of poultry would contain a mere 0.421 µg of phage particles, 0.284 mg of added sodium, and 0.007 mg of added potassium. Both the added sodium and potassium levels are so low as to not require any changes to labeling. The weight of added phage is negligible.

SalmoFresh™ is substantially equivalent to the lytic bacteriophage cocktails that have been previously designated GRAS and/or cleared by other regulatory agencies. Furthermore, with the proposed maximum application rate for SalmoFresh™ of up to 1×10^7 PFU per gram of food article, even in the worst case scenario (1×10^7 PFU/g,) the rate is much lower than the rates previously cleared for those other cocktails as safe and effective. For instance, the maximum proposed application rate of SalmoFresh™ is 100 times lower than that of the previously GRAS-listed Listex P100 bacteriophage preparation..

In summary, the data presented in this document fully supports our designation of SalmoFresh™ as GRAS. The basis for our conclusion is five-fold. First, the scientific literature extensively documents that lytic bacteriophages pose no safety concerns to humans. Second, All bacteriophages in SalmoFresh™ are lytic, non-genetically modified, and free of any and all undesirable genes. Third, Intralytix's manufacturing process ensures the safety and quality of the final SalmoFresh™ product. Fourth, the estimated daily intake of the SalmoFresh™ phage preparation is so low as to be negligible. And, fifth, the bacteriophage product is substantially equivalent to several bacteriophage products already receiving regulatory clearance, including GRN000218. Based on this information, it is evident that SalmoFresh™ is GRAS.

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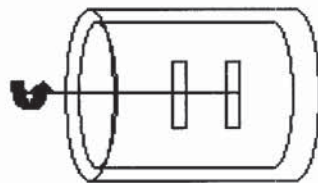
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6 APPENDICES

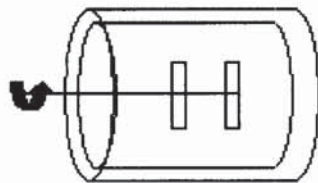
Appendix 1 Efficacy data

Added to fermentor:
-vegan broth
-antifoam (as needed)



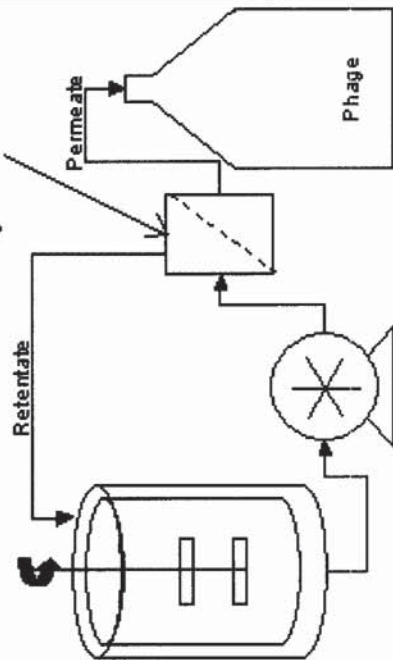
Sterilization of batch medium.

Added to fermentor:
-Bacterial host culture
-Monophage



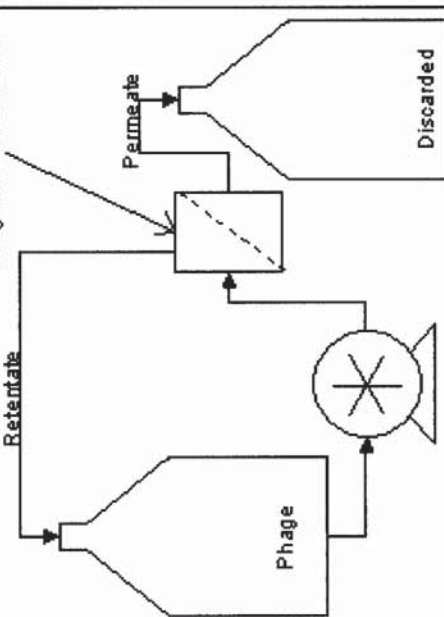
Inoculation, infection, and host lysis.

Tangential-flow filtration.



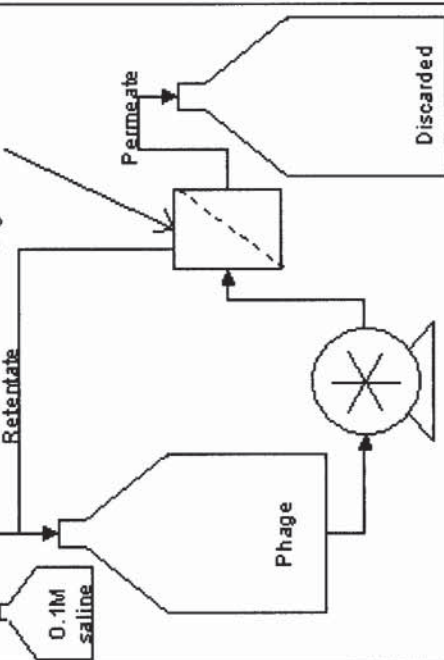
Clarification and removal of residual host bacteria. Retentate contains residual host; permeate contains phage. An optional centrifugation step may be added if necessary.

Tangential-flow filtration



Concentration of phage $\geq 10:1$ volume reduction. Retentate flow contains the monophage. Permeate flow contains smaller moieties.

Tangential-flow filtration



Washing & saline exchange. Retentate contains the monophage. Permeate contains smaller moieties. About 10:1 volume exchange with 0.1M saline.

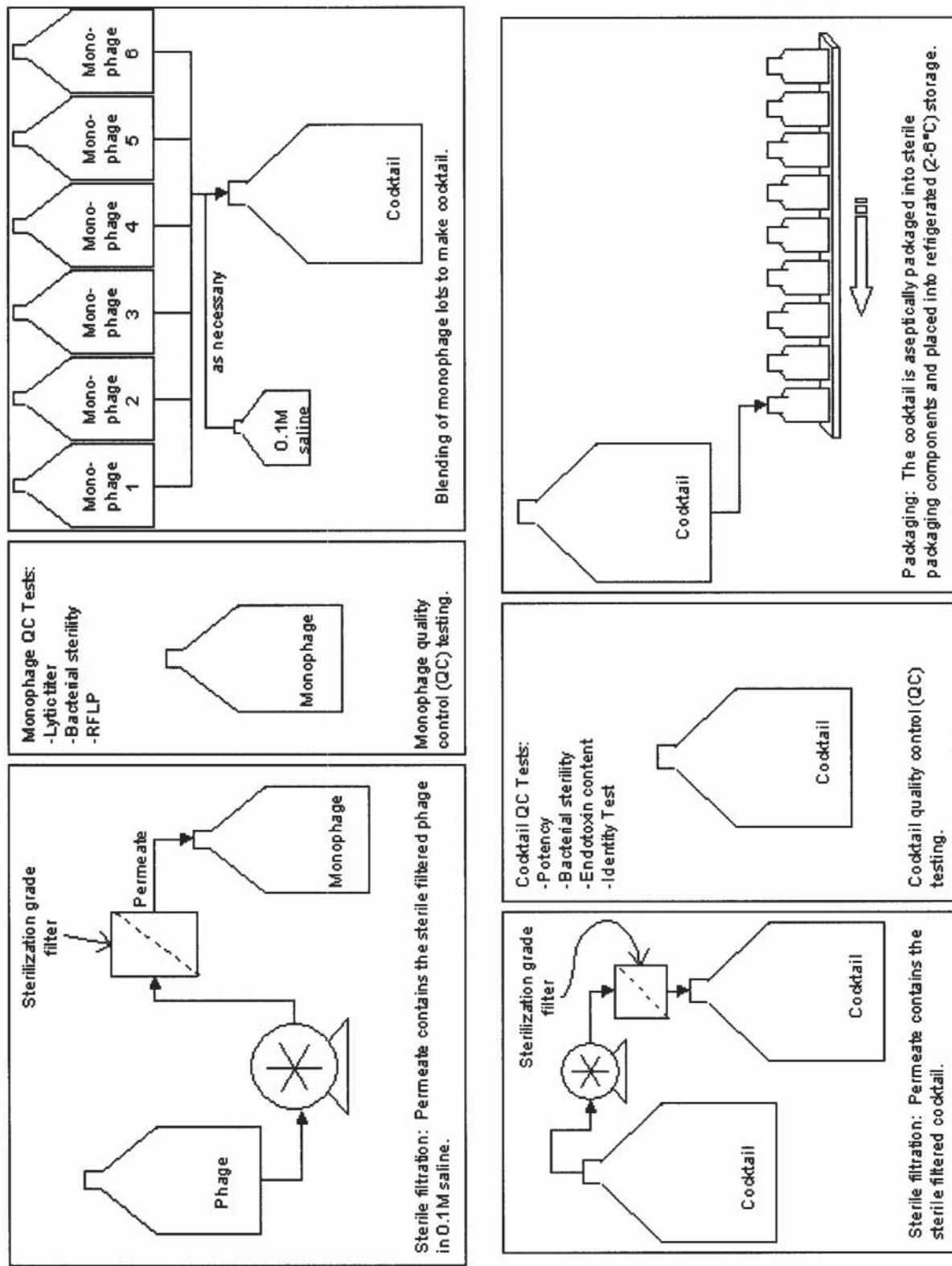


Figure 1 Overview of Salmofresh™ manufacturing process



Appendix 1: Efficacy

APPENDIX 1: EFFICACY STUDIES

It is requested that SalmoFresh™ be included in FSIS Directive 7120.1 as a safe and suitable antimicrobial used in the production of poultry products as a processing aid. As stated, its intended use is as a spray application for reducing levels of *Salmonella* when applied at the rate of ca. 1×10^6 – 1×10^7 PFU/g in ready-to-eat poultry prior to slicing or raw poultry prior to or after grinding.

Substance: Bacteriophage preparation (*Salmonella* targeted)

Product: Raw poultry prior to or after grinding and RTE poultry prior to slicing

Amount: Applied as a spray to the surface of the product at a level of ca. 1×10^6 – 1×10^7 plaque forming units (PFU) per gram of product

Reference: Acceptability determination

Labeling Requirements: None under the accepted conditions of use

SalmoFresh™ is an all-natural product made of six *Salmonella*-specific lytic bacteriophages from the *Myoviridae* family. All phages included in SalmoFresh™ are lytic phages that were obtained from the environment in the USA and have not been genetically manipulated in any way. The component phages of SalmoFresh™ have been rigorously characterized, including full genome sequencing.

The SalmoFresh™ preparation is intended for use in poultry products to control *Salmonella* when added in the range from 1×10^6 to 1×10^7 PFU per gram of food. SalmoFresh™ has been determined by Intralytix, Inc. to be generally recognized as safe (GRAS), and therefore, we believe it to be exempt from the requirement of pre-market approval, under the conditions of its intended use.

SALMOFRESH™ APPLICATION RATES AND DIETARY INTAKE

The proposed application rates of SalmoFresh™ are shown in Table 1. They are very similar to the application rates of two previously cleared bacteriophage preparations, ListShield™ and EcoShield™.

Based on the application rates, the estimated daily intake of SalmoFresh™ is also very safe and much lower than the intakes of other already cleared bacteriophage products. For example, assuming the worst case scenario where *all* turkey and chicken consumed in the US is treated with the *maximum amount* of SalmoFresh™, the daily intake of one person would be approximately 0.42 µg of phage per day. As a comparison, the FDA-cleared application rate for ListShield™ results in an intake of 1.3 µg of phage per day. The SalmoFresh™ daily intake is 67% less than that of ListShield™.

Table 1 Comparison of the proposed application rate of SalmoFresh™ and application rates of FDA-cleared ListShield™ and EcoShield™

Product	Test article	Reference	Phage concentration on food article (PFU/g)
SalmoFresh™	Turkey deli slices	Intralytix study: #SS11K08JW	4E+06
SalmoFresh™	Chicken deli slices	Intralytix study: #SS11K15JW	4E+06
SalmoFresh™	Turkey breast trim	Intralytix study: #SS11K09ML	9E+06 – 2E+07
SalmoFresh™	Turkey breast trim	Industry partner study: T3BT	4E+06 – 9E+06
SalmoFresh™	Dark ground turkey	Industry partner study: T4DG	4E+06 – 9E+06
SalmoFresh™	Dark ground turkey	Industry partner study: T2DG	1E+06
SalmoFresh™	Turkey breast trim	Industry partner study: T1BT	1E+06
SalmoFresh™	Turkey breast trim	Intralytix study: #SS11L19ML	9E+06
SalmoFresh™	Chicken breast	Intralytix study: #SS11L26ML	9E+06
ListShield™	Beef frankfurters	21 CFR §172.785*	3E+06
ListShield™	Ham	21 CFR §172.785	2E+06
ListShield™	Roast beef, uncured	21 CFR §172.785	2E+06
ListShield™	Sliced roast beef	21 CFR §172.785	2E+06
ListShield™	Turkey frankfurters	21 CFR §172.785	4E+06
ListShield™	Turkey pastrami	21 CFR §172.785	2E+06
ListShield™	Smoked turkey breast	21 CFR §172.785	2E+06
ListShield™	Roast turkey	21 CFR §172.785	2E+06
ListShield™	Sliced bologna, beef	21 CFR §172.785	3E+06
ListShield™	Sliced bologna, turkey	21 CFR §172.785	3E+06
ListShield™	Lebanon bologna	21 CFR §172.785	2E+06
ListShield™	Turkey salami	21 CFR §172.785	3E+06
EcoShield™	Beef slices	FCN No. 1018	3E+06 – 1E+07

* EcoLab Microbiological Services performed a contractual investigation of ListShield™ in July 2003 as Study Number 0300013, which was included in the FAP package submitted to the FDA for 21 CFR §172.785



SALMOFRESH™ IS EFFECTIVE.

Target range

SalmoFresh™ has been screened for its lytic activity against over 900 *Salmonella* strains represented by more than 50 serotypes. Of the fifteen serotypes that are represented by more than 5 strains each, SalmoFresh™ lyses 99-100% of Typhimurium, Enteritidis, Hadar, Heidelberg, Newport, Kentucky, Agona, Grampian, Senftenberg, Infantis, Reading and Thompson.

Effect on *Salmonella* levels in foods

Studies done by Intralytix and an industry partner demonstrate that SalmoFresh™ application at the proposed level of 1×10^6 – 1×10^7 PFU/g significantly reduces *Salmonella* levels in a variety of experimentally contaminated poultry foods. Nine studies are briefly summarized below:

- Study #SS11K08JW: SalmoFresh™, applied at 2 mL/lb (4×10^6 PFU/g) of ready-to-eat deli meat prior to slicing, significantly reduced *Salmonella* levels by 90% in oven-roasted turkey.
- Study #SS11K15JW: SalmoFresh™, applied at 2 mL/lb (4×10^6 PFU/g) of ready-to-eat deli meat prior to slicing, significantly reduced *Salmonella* levels by 98% in oven-roasted chicken.
- Study #SS11K09ML: SalmoFresh™, applied at 4 mL/lb (9×10^6 or 2×10^7 PFU/g) of raw turkey breast trim before grinding, significantly reduced *Salmonella* levels by 68-86%. Using 2-fold dilute SalmoFresh™ preparation (1×10^9 PFU/mL) vs. more concentrated SalmoFresh™ preparation (2×10^9 PFU/mL) did not significantly affect the efficacy.
- Study #T3BT: SalmoFresh™, applied at 2 or 4 mL/lb (4×10^6 or 9×10^6 PFU/g) of raw turkey breast trim before grinding, significantly reduced *Salmonella* levels 65-90% after 24-120 hours cold storage. Increased cold storage time showed no continued residual effect (i.e. no significant change in *Salmonella* levels after the initial reduction.)
- Study #T4DG: SalmoFresh™, applied at 2 or 4 mL/lb (4×10^6 or 9×10^6 PFU/g) of raw ground dark turkey, significantly reduced *Salmonella* levels 65-92% after 24-120 hours cold storage. Increased cold storage time showed no continued residual effect (i.e. no significant change in *Salmonella* levels after the initial reduction.)

- Study # T2DG: SalmoFresh™, applied at 0.5 mL/lb (1×10^6 PFU/g) of raw ground dark turkey, significantly reduced *Salmonella* levels 89-95% after 24-96 hours cold storage. Increased cold storage time showed no continued residual effect (i.e. no significant change in *Salmonella* levels after the initial reduction.)
- Study #T1BT: SalmoFresh™, applied at 0.5 mL/lb (1×10^6 PFU/g) of raw turkey breast trim before grinding, significantly reduced *Salmonella* levels 75-80% after 24-96 hours cold storage. Increased cold storage time showed no continued residual effect (i.e. no significant change in *Salmonella* levels after the initial reduction.)
- Study #SS11L19ML: SalmoFresh™, applied at 4 mL/lb (9×10^6 PFU/g) of raw turkey breast trim before grinding, significantly reduced *Salmonella* levels by 71%. SalmoFresh™ treatment did not have a residual protective effect in the ground meat; i.e., it did not significantly protect the ground turkey meat from subsequent recontamination with *Salmonella*.
- Study #SS11L26ML: SalmoFresh™, applied at 4 mL/lb (9×10^6 PFU/g) of raw chicken breast before grinding, significantly reduced *Salmonella* levels by 69%. SalmoFresh™ treatment did not have a residual protective effect in the ground meat; i.e., it did not significantly protect the ground chicken meat from subsequent recontamination with *Salmonella*.

SALMOFRESH™ IS A PROCESSING AID

SalmoFresh™ meets the statutory definition.

The FDA defines processing aids (in 21 CFR 101.100(a)(3)) as "substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food." SalmoFresh™ meets this definition, based on the following reasons:

- 1) SalmoFresh™ provides a momentary antimicrobial effect on treated poultry food products
- 2) SalmoFresh™ is present in the finished products at insignificant levels
- 3) SalmoFresh™ provides no continued technical effect

SalmoFresh™ is technically equivalent to other phage products designated as processing aids.

FSIS has already cleared several bacteriophage preparations as processing aids with no labeling requirements, including EcoShield™ and Listex™. SalmoFresh™ is

technically equivalent to those products. Furthermore, with the proposed application rate for SalmoFresh™ of $1 \times 10^6 - 1 \times 10^7$ PFU per gram of food article, even in the worst case scenario (1×10^7 PFU/g,) the rate is 100 times lower than the maximum 1×10^9 PFU/g for Listex™ P100 cleared by the FSIS as sufficiently low to not require labeling (FSIS Directive 7120.1). Table 2 below presents further comparisons the PFU/g of food article for SalmoFresh™ and those of previously cleared bacteriophage products with no labeling requirements. In all cases, the proposed SalmoFresh™ application rates are similar to or below the previously approved rates determined by the FSIS to not require labeling.

Table 2 Comparison of the phage concentration per gram of food article for SalmoFresh™ and other previously FDA-cleared bacteriophage products with no labeling requirements.

Product	Test article	Reference	Phage concentration on food article (PFU/g)
SalmoFresh™	Turkey deli slices	Intralytix study #SS11K08JW	4E+06
SalmoFresh™	Chicken deli slices	Intralytix study: #SS11K15JW	4E+06
SalmoFresh™	Turkey breast trim	Intralytix study: #SS11K09ML	9E+06 – 2E+07
SalmoFresh™	Turkey breast trim	Industry partner study: T3BT	4E+06 – 9E+06
SalmoFresh™	Dark ground turkey	Industry partner study: T4DG	4E+06 – 9E+06
SalmoFresh™	Dark ground turkey	Industry partner study: T2DG	1E+06
SalmoFresh™	Turkey breast trim	Industry partner study: T1BT	1E+06
SalmoFresh™	Turkey breast trim	Intralytix study: #SS11L19ML	9E+06
SalmoFresh™	Chicken breast	Intralytix study: #SS11L26ML	9E+06
Listex™P100	Cheese	GRAS Notification GRN 000218, pg 12 of 82	6E+08
Listex™P100	Ham	GRAS Notification GRN 000218, pg 78 of 82	3E+08
Listex™P100	Turkey breast	GRAS Notification GRN 000218, pg 79 of 82	3E+08
Listex™P100	Hot dogs	GRAS Notification GRN 000218, pg 79 of 82	3E+08
EcoShield™	Beef slices	FCN No. 1018	3E+06 – 1E+07

1.1 Description of intended technical effect

SalmoFresh™ is intended to produce a statistically significant reduction of *Salmonella* contamination vs. a water or carrier control when applied as directed to poultry products.

1.2 Efficacy study summary

SalmoFresh™ was examined for its ability to reduce *Salmonella* contamination when applied to cooked and raw poultry, prior to slicing, prior to grinding, and after grinding. Detailed reports of the studies are included in Appendix 1.1 - Appendix 1.8 and a summary of the results is given below.

1.2.1 Description of test systems

1.2.1.1 Deli meat

Precooked deli meat was inoculated with a mix of three *Salmonella* serotypes then treated with PBS or SalmoFresh™ at an application rate of 2 mL/lb of poultry. SalmoFresh™ contact was at room temperature for one hour, after which the samples were sliced and analyzed for populations of *Salmonella*.

1.2.1.2 Turkey breast trim

Uncooked turkey breast trim was inoculated with a mix of one-five *Salmonella* serotypes then treated with PBS or SalmoFresh™ at 4, 2 or 0.5 mL/lb of poultry. SalmoFresh™ contact time at was at room temperature for 5-30 minutes, after which the samples were ground. Samples were either immediately analyzed for populations of *Salmonella* or stuffed into casings and stored at 4°C until analyzed.

1.2.1.3 Ground dark turkey

Uncooked ground dark turkey was inoculated with a mix of five *Salmonella* serotypes then treated with SalmoFresh™ at 4, 2 or 0.5 mL/lb of poultry. SalmoFresh™ contact time at was at room temperature for 30 minutes, after which the samples were stuffed into casings and stored at 4°C until analyzed for populations of *Salmonella*.

1.2.1.4 Chicken breast

Uncooked chicken breasts were inoculated with a mix of three *Salmonella* serotypes then treated with PBS or SalmoFresh™ at 4 mL/lb of poultry. SalmoFresh™ contact time at was at room temperature for 5 minutes, after which the samples were ground. Samples were stored at 4°C until analyzed for populations of *Salmonella*.

1.2.2 Summary of results

Two types of deli meat, oven-roasted turkey and oven-roasted chicken, were examined; detailed reports of these two studies (SS11K08JW and SS11K15JW) are included in Appendix 1.1 and Appendix 1.2. SalmoFresh™ was applied at 2 mL/lb at the concentrations of 1×10^7 , 1×10^8 , and 1×10^9 PFU/mL. After one hour contact time, SalmoFresh™ significantly (by 90% and 98%,

respectively) reduced *Salmonella* contamination in oven-roasted turkey and oven-roasted chicken deli meat samples when applied at the rate of 2.0 mL SalmoFresh (ca. 1×10^9 PFU/mL) per lb poultry. In both studies, using higher (ca. 1×10^9 PFU/mL) concentration SalmoFresh™ resulted in statistically significantly better reduction in *Salmonella* levels compared to more dilute SalmoFresh™ (ca. 1×10^8 PFU/mL and 1×10^7 PFU/mL).

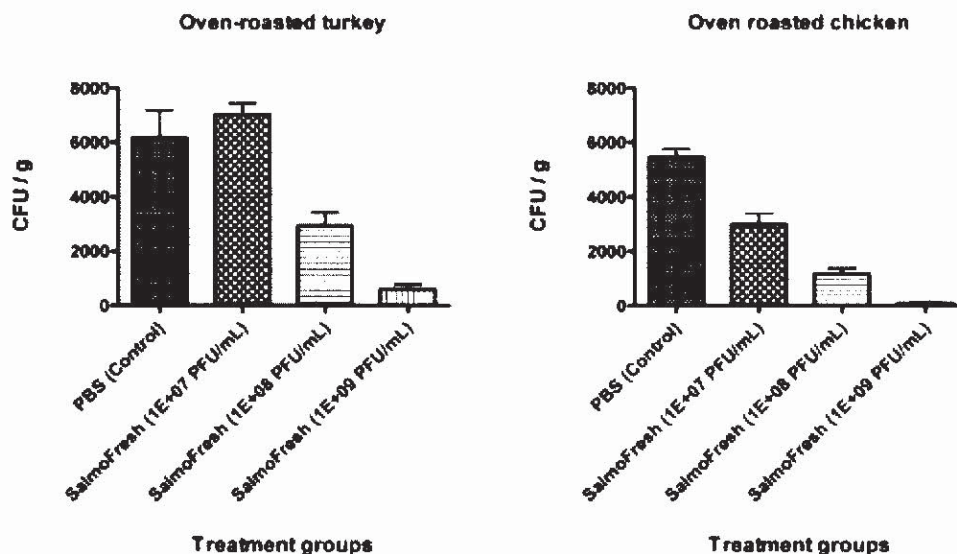


Figure 1 Reduction of *Salmonella* levels in deli meat samples treated with a range SalmoFresh™ concentrations at an application rate of 2 mL/lb (Studies SS11K08JW and SS11K15JW.)

Study SS11K09ML examined the efficacy of SalmoFresh™ on reducing *Salmonella* levels in uncooked turkey breast trim before being ground. Two concentrations of SalmoFresh™ (1×10^9 PFU/mL and 2×10^9 PFU/mL) were applied at a rate of 4 mL/lb to turkey breast five minutes prior to grinding and significantly reduced the number of viable *Salmonella* by ca. 68% and 86%, respectively (Table 3.) The complete details of this study can be seen in Appendix 1.3.

Table 3 Reduction of *Salmonella* levels in turkey breast trim treated with SalmoFresh™ at 4 mL/lb 5 minutes prior to grinding (Study SS11K09ML.)

SalmoFresh™ (PFU/mL)	Application rate (mL/lb)	Log ₁₀ reduction	% reduction	Significant
2×10^9	4.0	0.9	86	Yes
1×10^9	4.0	0.5	68	Yes

Further studies (T3BT, included in Appendix 1.4) examined the continued residual effect of SalmoFresh™ when applied to contaminated turkey breast trim 30 minutes prior to grinding, stuffing, and storage at 4°C. Treatment with SalmoFresh™, at 4 mL/lb and 2 mL/lb, significantly reduced *Salmonella* levels in turkey breast trim samples by an average of 82% and 73%,

respectively, but did not provide continued technical effect over the storage periods of 24-120 hours (i.e., effect is limited to the initial reduction and does not improve during storage). The higher application rate (4 mL/lb vs. 2 mL/lb) of SalmoFresh™ resulted in numerically better reduction in *Salmonella* levels; however, the differences were not statistically significant ($P > 0.05$).

To determine if the same application rates were effective on ground meat, SalmoFresh™ was applied to experimentally contaminated ground dark turkey meat, stuffed into casings, and stored at 4°C (study T4DG, included in Appendix 1.5.) Treatment with SalmoFresh™, at 4 mL/lb and 2 mL/lb, significantly reduced *Salmonella* levels in dark ground turkey by an average of 89% and 74%, respectively, but did not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage). Using higher application rate (4 mL/lb vs. 2 mL/lb) of SalmoFresh™ resulted in numerically better reduction in *Salmonella* levels in dark ground turkey. However, the differences were not statistically significant ($P > 0.05$).

As moisture content is often an issue of concern and the efficacy of the above application rates do not vary significantly, the efficacy of SalmoFresh™ at 0.5 mL/lb on ground dark turkey was examined (see study T2DG, included in Appendix 1.6.) SalmoFresh™ significantly (by 89, 93, and 95%) reduced *Salmonella* contamination in dark ground turkey samples stored for 24 h, 48 h, and 96 h, respectively, when applied at the rate of 0.5 mL SalmoFresh™ (ca. 1×10^9 PFU/mL) per lb of meat. Reduction in *Salmonella* levels at 24 h, 48 h, and 96 h were similar (differences not statistically significant ($P > 0.05$)). Summary: treatment with SalmoFresh™ can significantly reduce *Salmonella* levels in dark ground turkey samples by $\geq 89\%$, but it does not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage).

The efficacy of this 0.5 mL/lb application rate was also examined on turkey breast trim prior to grinding (see study T1BT, included in Appendix 1.7.) SalmoFresh™ significantly (by 74% and 79%) reduced *Salmonella* contamination in turkey breast trim samples stored for 24 h and 96 h, respectively, when applied at the rate of 0.5 mL SalmoFresh™ (ca. 1×10^9 PFU/mL) per lb of turkey meat. Reduction in *Salmonella* levels at 24 h and 96 h was similar (differences not statistically significant ($P > 0.05$)). Summary: treatment with SalmoFresh™ can significantly reduce *Salmonella* levels in turkey breast trim samples by $\geq 74\%$, but it does not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage).

Using the maximum application rate of 4 mL/lb of poultry, SalmoFresh™ was applied to experimentally contaminated turkey breast trim prior to grinding (see study SS11L19ML, included in Appendix 1.8.) Samples were stored for 24 hours before being re-contaminated with *Salmonella*. Similar to the studies above, SalmoFresh™ significantly reduced the original *Salmonella* contamination by 71%. However, after recontamination, the levels of *Salmonella* recovered from SalmoFresh™-treated or untreated samples were similar (statistically not different.) Summary: treatment with SalmoFresh™ can significantly reduce *Salmonella* levels in turkey breast trim samples by ca 71%, but it does not provide continued technical effect.

Again using the maximum application rate of 4 mL/lb of poultry, SalmoFresh™ was applied to experimentally contaminated chicken breast prior to grinding (see study SS11L26ML, included in Appendix 1.8). Samples were stored for 24 hours before being re-contaminated with *Salmonella*. SalmoFresh™ significantly reduced the original *Salmonella* contamination by 69%.

After recontamination, the levels of *Salmonella* recovered from SalmoFresh™-treated or untreated samples were similar (statistically not different.) Summary: treatment with SalmoFresh™ can significantly reduce *Salmonella* levels in chicken breast samples by ca 69%, but it does not provide continued technical effect.

Table 4 summarizes the data from the deli meat, turkey breast trim, ground dark turkey meat, and chicken breast studies. The data shows that SalmoFresh™, when applied to poultry products before slicing, before grinding, or after grinding at a rate of $1 \times 10^6 - 1 \times 10^7$ PFU/g poultry product, can reduce contamination of poultry products by 65-98%. Additionally, after the initial reduction of *Salmonella* contamination, the levels of *Salmonella* contamination remain statistically the same. Therefore, after its initial momentary reduction of *Salmonella* contamination, SalmoFresh™ provides no continued technical effect.

1.2.3 Summary

We believe the data summarized here fully supports our request for SalmoFresh™ to be included in FSIS directive 7120.1 as a safe and suitable ingredient used in the production of poultry products as a processing aid. Its intended use is as a spray applied to significantly reduce levels of *Salmonella* when applied at ca. $1 \times 10^6 - 1 \times 10^7$ PFU/g in ready-to-eat poultry prior to slicing, raw poultry prior to grinding, or raw poultry after grinding. Additionally, no foods treated to product specifications should require SalmoFresh™ as a listed ingredient on product labels.

1.3 Appendices

Appendix 1.1	<u>SS11K08JW</u>
Appendix 1.2	<u>SS11K15JW</u>
Appendix 1.3	<u>SS11K09ML</u>
Appendix 1.4	<u>T3BT</u>
Appendix 1.5	<u>T4DG</u>
Appendix 1.6	<u>T2DG</u>
Appendix 1.7	<u>T1BT</u>
Appendix 1.8	<u>SS11L19ML</u>
Appendix 1.9	<u>SS11L26ML</u>

Table 4 Reduction in *Salmonella* levels in experimentally contaminated poultry products treated with 1×10^9 PFU/mL SalmoFresh™ at three application rates.

Study #	Matrix	Application rate (mL/lb)	Storage at 4°C (hrs)	Log ₁₀ reduction	% reduction	Significant?	Continued residual effect?
SS11K08JW	Turkey deli meat	2	n/a	1.0	90	Yes	n/a
SS11K15JW	Chicken deli meat	2	n/a	1.7	98	Yes	n/a
SS11K09ML	Turkey breast trim	4	n/a	0.5	68	Yes	n/a
T3BT	Turkey breast trim	4	24	0.6	77	Yes	n/a
			48	1.0	90	Yes	No
			72	0.6	78	Yes	No
			120	0.8	83	Yes	No
	Turkey breast trim	2	24	0.5	65	Yes	n/a
			48	0.7	79	Yes	No
			72	0.5	68	Yes	No
			120	0.7	80	Yes	No
T4DG	Ground dark turkey	4	24	0.8	83	Yes	n/a
			48	1.1	92	Yes	No
			72	1.0	90	Yes	No
			120	1.0	90	Yes	No
	Ground dark turkey	2	24	0.6	75	Yes	n/a
			48	0.6	72	Yes	No
			72	0.5	65	Yes	No
			120	0.8	83	Yes	No
T2DG	Ground dark turkey	0.5	24	0.9	89	Yes	n/a
			48	1.2	93	Yes	No
			96	1.3	95	Yes	No
T1BT	Turkey breast trim	0.5	24	0.6	75	Yes	n/a
			96	0.7	80	Yes	No
SS11L19ML	Turkey breast trim	4	24	0.5	71	Yes	No
SS11L26ML	Chicken breast	4	24	0.5	69	Yes	No

Appendix 1.1:
Study #SS11K08JW





Evaluation of the ability of different concentrations of SalmoFresh to reduce *Salmonella* contamination in experimentally contaminated oven-roasted turkey when applied at the rate of 2.0 mL per lb of poultry.

Study # SS11K08JW



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1 STUDY TITLE

Evaluation of the ability of different concentrations of SalmoFresh™ to reduce *Salmonella* contamination in experimentally contaminated oven-roasted turkey when applied at the rate of 2.0 mL per lb of poultry.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Joelle Woolston, M.S.	Research Scientist	Hands-on-research

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of different concentrations of SalmoFresh™ reduces the number of viable *Salmonella* in oven roasted turkey deli meat when applied at the rate of 2mL per lb of poultry.

6 TEST MATRIX

A sample of oven-roasted turkey was purchased from a local grocery store deli meat counter in Baltimore, MD. It was not washed or pre-treated prior to our studies.

7 SALMOFRESH LOT AND APPLICATION RATE

- SalmoFresh™ Lot #02TestSample
- Titer: approx. 1×10^7 PFU/mL, 1×10^8 PFU/mL, and 1×10^9 PFU/mL
- SalmoFresh™ was applied using Basic Spray Gun Model #200 (Badger Air-Brush Co., Franklin Park, IL).
- The application rate was ca. 2mL SalmoFresh™ per 1 pound of poultry.

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE POULTRY

The poultry test matrix was experimentally contaminated with a 1:1:1 mixture of three *Salmonella* strains:

- *S.E900*: A nalidixic acid resistant mutant developed from *S.E660* (also known as ATCC13076, *Salmonella enterica* serotype Enteritidis.)
- *S.Ty901*: A nalidixic acid resistant mutant developed from *S.Ty653* (also known as ATCC6539, *Salmonella enterica* serotype Typhi.)
- *S.He902*: A nalidixic acid resistant mutant developed from *S.He899* (also known as ATCC8326, *Salmonella enterica* serotype Heidelberg.)

The strains were selected for nalidixic acid resistance by serially passaging the original isolates on LB agar plates supplemented with increasing concentrations of nalidixic acid. Each strain underwent ≤ 8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 $\mu\text{g/ml}$. After the passaging, the above-noted Intralytix strain designations were assigned (i.e., *S.E900*, *S.Ty901*, and *S.He902*). The strains were stored at -80°C , at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 μg of nalidixic acid/ml.

Shortly before performing the study, the three strains were thawed and grown ($37 \pm 2^{\circ}\text{C}$, 24-48 h) in NZCYM broth supplemented with nalidixic acid (25 $\mu\text{g/ml}$) until the cultures reached an OD_{600} of ca. 1.5, which corresponds to ca. 1×10^9 CFU/mL. Equal volumes of three bacterial cultures were mixed and the mixture diluted 100-fold just prior to performing the study.

The turkey was experimentally contaminated by ca. 25,000 CFU of the above-defined 1:1:1 mixture of three *Salmonella* strains / g of turkey.

9 MEDIA AND REAGENTS

- NZCYM (Becton, Dickinson and Co., Sparks, MD; cat #215251)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Salmonella/Shigella Agar (SSA) (Becton, Dickinson and Co., Sparks, MD; cat #274500)

10 GENERAL OUTLINE OF STUDY

- 1) The challenge dose of bacteria was applied onto the matrix samples' surfaces. Bacterial cultures were evenly spread onto all sides of the poultry sample surfaces using hockey sticks.
- 2) The bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
- 3) PBS (control) or SalmoFresh™ was applied as described in section 7. Poultry samples were rotated and all sides of the samples were sprayed, to ensure reasonably even coverage of the entire surface.
- 4) The samples were covered and incubated at room temperature for ca. 60 minutes.

- 5) At 60 minutes post-treatment with PBS or SalmoFresh™, two slices were cut off the end using a food slicer (Nesco catalog #FS-150PR) and discarded. The remaining sample was sliced until approximately 0.3 inches remained.
- 6) For each treatment group, ~25g replicates of slices were placed into sterile bags and ~225 mL of sterile peptone water was added. The bags were shaken by hand for 30 seconds.
 - The actual sample weight and peptone water volume were noted for each replicate.
- 7) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.3 mL) of the hand-mixed meat/peptone water mixture onto separate SSA plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\text{Total CFU}}{\text{g of poultry}} = \frac{\text{actual CFU}}{0.1\text{mL plating}} \times \frac{\text{actual mL peptone}}{\text{actual g sample}}$$

Counts from 0.1 mL plating were used during the analysis, because they provided most robust, countable numbers (i.e., more than 10 whenever possible but less than 100 colonies per plate)

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study # SS11K08JW

Sample	Weight (g)	Bacteria	Treatment	25g replicate samples	CFU in 0.1 ml	CFU/g
1	173	Yes	PBS	4	75; 62; 50; 101	5784; 5382; 4421; 9047
2	197	Yes	1x10 ⁷ PFU/mL SalmoFresh™	4	78; 99; 94; 86	6299; 7747; 7724; 6253
3	220	Yes	1x10 ⁸ PFU/mL SalmoFresh™	4	23; 33; 34; 46	1763; 2881; 2944; 4184
4	239	Yes	1x10 ⁹ PFU/mL SalmoFresh™	4	4; 11; 10; 3	357; 994; 892; 258

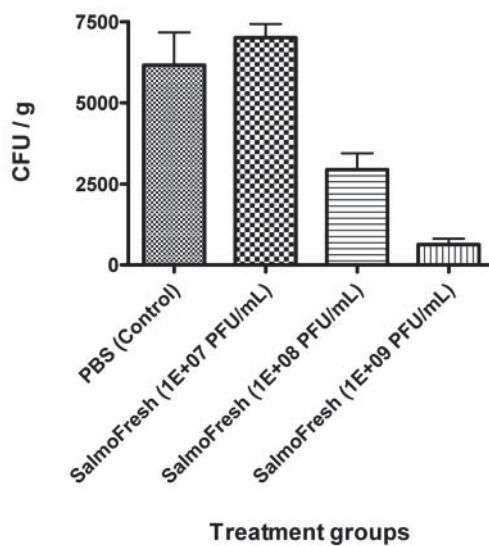
11.2 Tabular presentation of results

Table 2 Reduction of *Salmonella* counts in oven roasted turkey treated with ca. 1×10^7 , 1×10^8 , and 1×10^9 PFU/mL SalmoFresh™ when applied at the rate of 2mL per lb of poultry.

Challenged with <i>Salmonella</i>	Treatment	Replicates	Mean CFU/g	Percent reduction vs. PBS	Significant?
Yes	PBS	Control $n=4$	6158		
Yes	1×10^7 PFU/mL SalmoFresh™	$n=4$	7006	-14%	No
Yes	1×10^8 PFU/mL SalmoFresh™	$n=4$	2943	52%	Yes
Yes	1×10^9 PFU/mL SalmoFresh™	$n=4$	625	90%	Yes

11.3 Graphical presentation of efficacy of results

Chart constructed using raw data (mean with SEM)



11.4 Statistical analysis

The efficacy of the SalmoFresh treatment in reducing the number of viable *Salmonella* in the experimentally contaminated turkey was evaluated by comparing the data obtained with the PBS-treated control samples and the SalmoFresh-treated samples.

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
PBS (Control) vs SalmoFresh (1E+07 PFU/mL)	-847.25	1.400	ns P>0.05
PBS (Control) vs SalmoFresh (1E+08 PFU/mL)	3215.5	5.312	* P<0.05
PBS (Control) vs SalmoFresh (1E+09 PFU/mL)	5533.3	9.141	*** P<0.001
SalmoFresh (1E+07 PFU/mL) vs SalmoFresh (1E+08 PFU/mL)	4062.8	6.712	** P<0.01
SalmoFresh (1E+07 PFU/mL) vs SalmoFresh (1E+09 PFU/mL)	6380.5	10.541	*** P<0.001
SalmoFresh (1E+08 PFU/mL) vs SalmoFresh (1E+09 PFU/mL)	2317.8	3.829	ns P>0.05

11.5 Brief discussion of results and study's conclusions

- Applying ca. 1×10^9 PFU/mL SalmoFresh™ to oven roasted turkey meat – at the rate of 2.0 mL per lb of poultry - reduced the number of viable *Salmonella* by ca. 90% after ca. 60 minutes of incubation at room temperature. The observed reduction was statistically significant ($P < 0.001$).
- Applying ca. 1×10^8 PFU/mL SalmoFresh™ to oven roasted turkey meat – at the rate of 2.0 mL per lb of poultry - reduced the number of viable *Salmonella* by ca. 52% after ca. 60 minutes of incubation at room temperature. The observed reduction was statistically significant ($P < 0.05$).
- Applying 1×10^7 PFU/mL SalmoFresh™ to oven roasted turkey meat – at the rate of 2.0 mL per lb of poultry – did not reduce the number of viable *Salmonella* after ca. 60 minutes of incubation at room temperature (difference statistically not significant, $P > 0.05$).
- Reduction in *Salmonella* levels achieved by using more concentrated SalmoFresh™ was higher compared to those obtained with more dilute SalmoFresh™ (90% vs. 52% vs. 0% reduction when using ca. 1×10^9 PFU/mL, 1×10^8 PFU/mL and ca. 1×10^7 PFU/mL of SalmoFresh™, respectively).
- The difference in *Salmonella* recovery between SalmoFresh™ 10^9 PFU/mL treated samples vs. SalmoFresh™ 10^7 PFU/mL treated samples, and SalmoFresh™ 10^8

PFU/mL treated samples vs. SalmoFresh™ 10^7 PFU/mL treated samples were statistically significant.

- The difference in *Salmonella* recovery between SalmoFresh™ 10^9 PFU/mL treated samples vs. SalmoFresh™ 10^8 PFU/mL treated samples were statistically not significant.

12 SUMMARY CONCLUSION OF THE STUDY

SalmoFresh™ significantly (by 90%) reduced *Salmonella* contamination in turkey meat samples when applied at the rate of 2.0 mL SalmoFresh (ca. 1×10^9 PFU/mL) per lb of turkey meat.

Using higher (ca. 1×10^9 PFU/mL and 1×10^8 PFU/mL) concentrations of SalmoFresh™ resulted in statistically significantly better reduction in *Salmonella* levels compared to more dilute SalmoFresh™ (ca. 1×10^7 PFU/mL).

13 SIGNATURES

(b) (6)



Joelle Woolston, M.S.
Research Scientist

December 15, 2011

(b) (6)



Alexander Sulakvelidze, Ph.D.
Study Director

December 15, 2011

Appendix 1.2:
Study #SS11K15JW





Evaluation of the ability of different concentrations of SalmoFresh to reduce *Salmonella* contamination in experimentally contaminated oven-roasted chicken when applied at the rate of 2.0 mL per lb of poultry.

Study # SS11K15JW

Intraluxt, Inc.

The Columbus Center

701 E. Pratt St.

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www.intraluxt.com

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1 STUDY TITLE

Evaluation of the ability of different concentrations of SalmoFresh™ to reduce *Salmonella* contamination in experimentally contaminated oven-roasted chicken when applied at the rate of 2.0 mL per lb of poultry.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Joelle Woolston, M.S.	Research Scientist	Hands-on-research

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine the efficacy of different concentrations of SalmoFresh™ on reducing the number of viable *Salmonella* in oven-roasted chicken deli meat, when applied at the rate of 2mL per lb of poultry.

6 TEST MATRIX

A sample of oven-roasted chicken was purchased from a local grocery store deli meat counter in Baltimore, MD. It was not washed or pre-treated prior to our studies.

7 SALMOFRESH LOT AND APPLICATION RATE

- SalmoFresh™ Lot #02TestSample
- Titer: approx. 1×10^7 PFU/mL, 1×10^8 PFU/mL, and 1×10^9 PFU/mL
- SalmoFresh™ was applied using Basic Spray Gun Model #200 (Badger Air-Brush Co., Franklin Park, IL).
- The application rate was ca. 2mL SalmoFresh™ per 1 pound of poultry.

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE POULTRY

The poultry test matrix was experimentally contaminated with a 1:1:1 mixture of three *Salmonella* strains:

- *S.E900*: A nalidixic acid resistant mutant developed from *S.E660* (also known as ATCC13076, *Salmonella enterica* serotype Enteritidis.)
- *S.Ty901*: A nalidixic acid resistant mutant developed from *S.Ty653* (also known as ATCC6539, *Salmonella enterica* serotype Typhi.)
- *S.He902*: A nalidixic acid resistant mutant developed from *S.He899* (also known as ATCC8326, *Salmonella enterica* serotype Heidelberg.)

The strains were selected for nalidixic acid resistance by serially passaging the original isolates on LB agar plates supplemented with increasing concentrations of nalidixic acid. Each strain underwent ≤ 8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 $\mu\text{g/ml}$. After the passaging, the above-noted Intralytix strain designations were assigned (i.e., *S.E900*, *S.Ty901*, and *S.He902*). The strains were stored at -80°C , at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 μg of nalidixic acid/ml.

Shortly before performing the study, the three strains were thawed and grown ($37 \pm 2^{\circ}\text{C}$, 24-48 h) in NZCYM broth supplemented with nalidixic acid (25 $\mu\text{g/ml}$) until the cultures reached an OD_{600} of ca. 1.5, which corresponds to ca. 1×10^9 CFU/mL. Equal volumes of three bacterial cultures were mixed and the mixture diluted 100-fold just prior to performing the study.

The chicken was experimentally contaminated by ca. 25,000 CFU of the above-defined 1:1:1 mixture of three *Salmonella* strains / g of chicken.

9 MEDIA AND REAGENTS

- NZCYM (Becton, Dickinson and Co., Sparks, MD; cat #215251)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Salmonella/Shigella Agar (SSA) (Becton, Dickinson and Co., Sparks, MD; cat #274500)

10 GENERAL OUTLINE OF STUDY

- 1) The challenge dose of bacteria was applied onto the matrix samples' surfaces. Bacterial cultures were evenly spread onto all sides of the poultry sample surfaces using hockey sticks.
- 2) The bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
- 3) PBS (control) or SalmoFresh™ was applied as described in section 7. Poultry samples were rotated and all sides of the samples were sprayed, to ensure reasonably even coverage of the entire surface.
- 4) The samples were covered and incubated at room temperature for ca. 60 minutes.

- 5) At 60 minutes post-treatment with PBS or SalmoFresh™, two slices were cut off the end using a food slicer (Nesco catalog #FS-150PR) and discarded. The remaining sample was sliced until approximately 0.3 inches remained.
- 6) For each treatment group, ~25g replicates of slices were placed into sterile bags and ~225 mL of sterile peptone water was added. The bags were shaken by hand for 30 seconds.
 - The actual sample weight and peptone water volume were noted for each replicate.
- 7) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.3 mL) of the hand-mixed meat/peptone water mixture onto separate SSA plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\text{Total CFU}}{\text{g of poultry}} = \frac{\text{actual CFU}}{0.1\text{mL plating}} \times \frac{\text{actual mL peptone}}{\text{actual g sample}}$$

Note: Counts from 0.1 mL plating were used during the analysis, because they provided most robust, countable numbers (i.e., more than 10 whenever possible but less than 100 colonies per plate)

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #SS11K15JW

Sample	Weight (g)	Bacteria	Treatment	25g replicate samples	CFU in 0.1 ml	CFU/g
1	173	Yes	PBS	4	67; 59; 55; 50	5739; 4810; 5152; 6151
2	197	Yes	1x10 ⁷ PFU/mL SalmoFresh™	4	48; 42; 26; 32	3980; 3342; 2151; 2482
3	220	Yes	1x10 ⁸ PFU/mL SalmoFresh™	5	18; 8; 15; 8; 17	1432; 723; 1305; 708; 1733
4	239	Yes	1x10 ⁹ PFU/mL SalmoFresh™	5	1; 0; 3; 0; 2	77; 0; 266; 0; 146

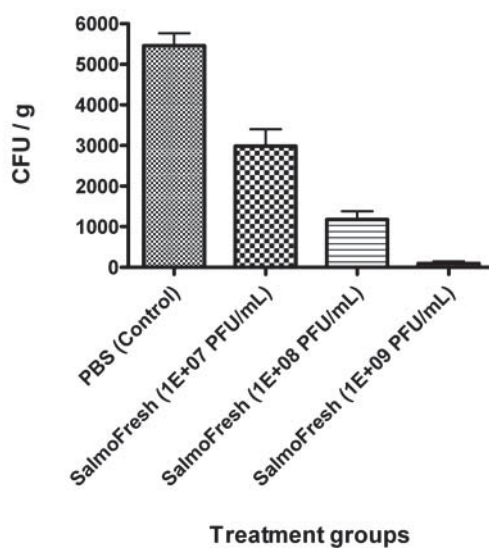
11.2 Tabular presentation of results

Table 2 Reduction of *Salmonella* counts in oven-roasted chicken treated with ca. 1×10^7 , 1×10^8 , and 1×10^9 PFU/mL SalmoFresh™ when applied at the rate of 2mL per lb of poultry.

Challenged with <i>Salmonella</i>	Treatment	Replicates	Mean CFU/g	Percent reduction vs. PBS	Significant?
Yes	PBS	Control $n=4$	5463		
Yes	1×10^7 PFU/mL SalmoFresh™	$n=4$	2989	45%	No
Yes	1×10^8 PFU/mL SalmoFresh™	$n=5$	1180	78%	Yes
Yes	1×10^9 PFU/mL SalmoFresh™	$n=5$	98	98%	Yes

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)



11.4 Statistical analysis

The efficacy of the SalmoFresh treatment in reducing the number of viable *Salmonella* in the experimentally contaminated chicken was evaluated by comparing the data obtained with the PBS-treated control samples and the SalmoFresh-treated samples.

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
PBS (Control) vs SalmoFresh (1E+07 PFU/mL)	2474.3	9.252	*** P<0.001
PBS (Control) vs SalmoFresh (1E+08 PFU/mL)	4282.8	16.880	*** P<0.001
PBS (Control) vs SalmoFresh (1E+09 PFU/mL)	5365.2	21.146	*** P<0.001
SalmoFresh (1E+07 PFU/mL) vs SalmoFresh (1E+08 PFU/mL)	1808.6	7.128	*** P<0.001
SalmoFresh (1E+07 PFU/mL) vs SalmoFresh (1E+09 PFU/mL)	2891.0	11.394	*** P<0.001
SalmoFresh (1E+08 PFU/mL) vs SalmoFresh (1E+09 PFU/mL)	1082.4	4.525	* P<0.05

11.5 Brief discussion of results and study's conclusions

- Applying ca. 1×10^9 PFU/mL SalmoFresh™ to oven-roasted chicken meat – at the rate of 2.0 mL per lb of poultry - reduced the number of viable *Salmonella* by ca. 98% after ca. 60 minutes of incubation at room temperature. The observed reduction was statistically significant ($P < 0.001$).
- Applying ca. 1×10^8 PFU/mL SalmoFresh™ to oven-roasted chicken meat – at the rate of 2.0 mL per lb of poultry - reduced the number of viable *Salmonella* by ca. 78% after ca. 60 minutes of incubation at room temperature. The observed reduction was statistically significant ($P < 0.001$).
- Applying 1×10^7 PFU/mL SalmoFresh™ to oven-roasted chicken meat – at the rate of 2.0 mL per lb of poultry – reduced the number of viable *Salmonella* by ca. 45% after ca. 60 minutes of incubation at room temperature. The observed reduction was statistically significant ($P < 0.001$).
- Reduction in *Salmonella* levels achieved by using more concentrated SalmoFresh™ was higher compared to those obtained with more dilute SalmoFresh™ (98% vs. 78% vs. 45% reduction when using ca. 1×10^9 PFU/mL, 1×10^8 PFU/mL and ca. 1×10^7 PFU/mL of SalmoFresh™, respectively).
- The differences in *Salmonella* recovery between various concentrations of SalmoFresh™ (10^9 PFU/mL vs. 10^8 PFU/mL vs. 10^7 PFU/mL) were statistically significant ($P < 0.001$)

12 SUMMARY CONCLUSION OF THE STUDY

SalmoFresh™ (ca. 1×10^9 PFU/mL) significantly (by 98%) reduced *Salmonella* contamination in chicken meat samples when applied at the rate of 2.0 mL SalmoFresh per lb of chicken meat.

Using higher (ca. 1×10^9 PFU/mL) concentration SalmoFresh™ resulted in statistically significantly better reduction in *Salmonella* levels compared to more dilute SalmoFresh™ (ca. 1×10^8 PFU/mL and 1×10^7 PFU/mL).

13 SIGNATURES

(b) (6)



Joelle Woolston, M.S.
Research Scientist

December 15, 2011

(b) (6)



Alexander Sulakvelidze, Ph.D.
Study Director

December 15, 2011

Appendix 1.3:
Study #SS11K09ML





**Evaluation of the ability of SalmoFresh to
reduce *Salmonella* contamination in
experimentally contaminated turkey trim when
applied at the rate of 4.0 mL per lb of poultry
prior to grinding.**

Study # SS11K09ML

Intralysix

The Columbus Center

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1 STUDY TITLE

Evaluation of the ability of SalmoFresh™ to reduce *Salmonella* contamination in experimentally contaminated turkey trim when applied at the rate of 4.0 mL per lb of poultry prior to grinding.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Manrong Li, MD, MS.	Research Scientist	Hands-on-research

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of SalmoFresh™ reduces the number of viable *Salmonella* in ground turkey when applied at the rate of 4mL per lb of poultry prior to grinding.

6 TEST MATRIX

A sample of turkey trim was obtained from [REDACTED] It was not washed or pre-treated prior to our studies.

7 SALMOFRESH LOT AND APPLICATION RATE

- SalmoFresh™ Lot #02TestSample
- Titer: approx. 2×10^9 PFU/mL and 1×10^9 PFU/mL
- SalmoFresh™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).
- The application rate was ca. 4mL SalmoFresh™ per 1 pound of poultry.

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE POULTRY

The poultry test matrix was experimentally contaminated with a single *Salmonella* strain:

- *S.He902*: A nalidixic acid resistant mutant developed from *S.He899* (also known as ATCC8326, *Salmonella enterica* serotype Heidelberg).

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤ 8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 $\mu\text{g/ml}$. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., *S.He902*). The strain was stored at -80°C , at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 μg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown ($37 \pm 2^{\circ}\text{C}$, 24-48 h) in NZCYM broth supplemented with nalidixic acid (25 $\mu\text{g/ml}$) until the culture reached an OD_{600} of ca. 1.5, which corresponds to ca. 1×10^9 CFU/mL. The bacterial culture was diluted 1000-fold just prior to performing the study.

The turkey was experimentally contaminated by ca. 1,250 CFU of the above-defined *Salmonella* culture / g of turkey trim.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Salmonella/Shigella Agar (SSA) (Becton, Dickinson and Co., Sparks, MD; cat #274500)

10 GENERAL OUTLINE OF STUDY

- 1) The challenge dose of bacteria was applied onto the matrix samples' surfaces. Bacterial cultures were evenly spread onto all sides of the poultry sample surfaces using hockey sticks. One sample was not treated with bacterial cultures as the uncontaminated, untreated control.
- 2) The bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
- 3) PBS (control) or SalmoFresh™ was applied as described in section 7. Samples in Group A were treated with 2×10^9 PFU/mL, and samples in Group B were treated with 1×10^9 PFU/mL of SalmoFresh™. Poultry samples were rotated and all sides of the samples were sprayed, to ensure reasonably even coverage of the entire surface.
- 4) The samples were covered and incubated at room temperature for ca. 5 minutes.
- 5) At 5 minutes post-treatment with water or SalmoFresh™, all samples were ground using a #10 meat grinder (Kitchener #508313).
- 6) Directly after grinding, from each sample group, triplicate ~25g samples of ground meat were removed, placed into sterile bags, and 225 mL of sterile peptone water was

added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds.

- 7) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.3 mL) of the stomached meat/peptone water mixture onto separate SSA plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated ($35 \pm 2^\circ\text{C}$, 24 ± 2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\text{Total CFU/g of treated poultry} = \text{CFU} / \text{mL plated} \times \text{mL peptone water} : \text{g sample analyzed}$$

Counts from 0.3 mL plating were used during the analysis, because they provided most robust, countable numbers (i.e., more than 10 whenever possible but less than 100 colonies per plate).

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #SS11K09ML

Group	Weight (g)	Bacteria	Treatment	25g Samples	CFU in 0.3 mL	CFU/g
A (Test)	200	Yes	2×10^9 PFU/mL SalmoFresh™	3	2; 5; 3	60; 150; 90
B (Test)	200	Yes	1×10^9 PFU/mL SalmoFresh™	3	7; 8; 8	210; 240; 240
C (Control)	200	Yes	PBS	3	27; 22; 24	810; 660; 720

11.2 Tabular presentation of results

Table 2 Reduction of *Salmonella* counts in ground turkey treated with ca. 2×10^9 PFU/mL and 1×10^9 PFU/mL SalmoFresh™ when applied at the rate of 4mL per lb of poultry prior to grinding.

Group	Challenged with <i>Salmonella</i>	Treatment	Replicates	Mean CFU/g	Percent reduction vs. PBS	Significant?
A (Test)	Yes	2×10^9 PFU/mL SalmoFresh™	$n = 3$	100	86.3%	Yes
B (Test)	Yes	1×10^9 PFU/mL SalmoFresh™	$n = 3$	230	68.5%	Yes
C (Control)	Yes	PBS	$n = 3$	730		

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

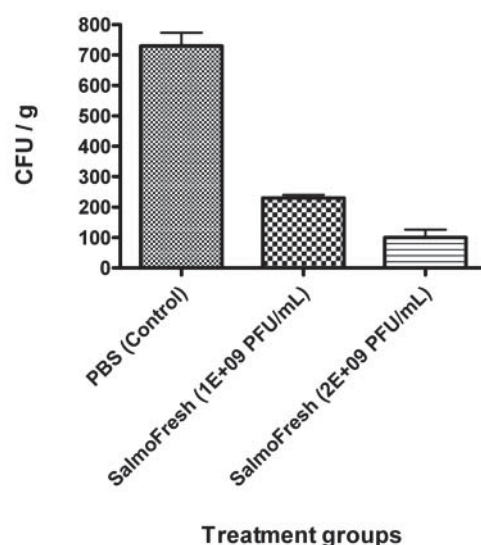
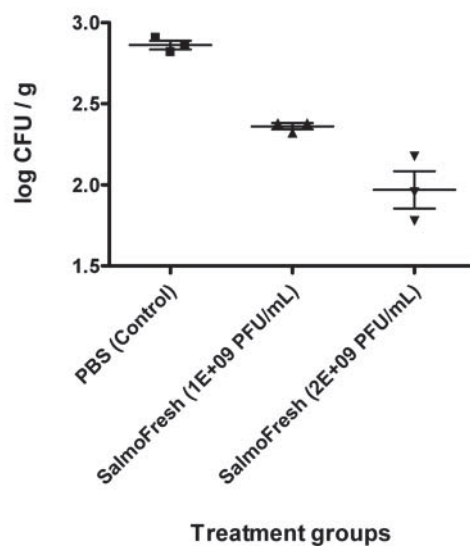


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the SalmoFresh™ treatment in reducing the number of viable *Salmonella* in the experimentally contaminated turkey samples was evaluated by comparing the data obtained with the PBS-treated control samples and the SalmoFresh™-H and SalmoFresh™-L treated samples.

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
PBS (Control) vs SalmoFresh (1E+09 PFU/mL)	500.00	16.667	*** P<0.001
PBS (Control) vs SalmoFresh (2E+09 PFU/mL)	630.00	21.000	*** P<0.001
SalmoFresh (1E+09 PFU/mL) vs SalmoFresh (2E+09 PFU/mL)	130.00	4.333	ns P>0.05

11.5 Brief discussion of results and study's conclusions

- Applying 2×10^9 PFU/mL SalmoFresh™ to turkey trim prior to grinding – at the rate of 4.0 mL per lb of poultry - reduced the number of viable *Salmonella* by ca. 86% after ca. 5 minutes of incubation at room temperature. The observed reduction was statistically significant ($P = <0.001$).
- Applying 1×10^9 PFU/mL SalmoFresh™ to turkey trim prior to grinding – at the rate of 4.0 mL per lb of poultry - reduced the number of viable *Salmonella* by ca. 69% after ca. 5 minutes of incubation at room temperature. The observed reduction was statistically significant ($P = <0.001$).
- The results obtained by treating the contaminated meat with SalmoFresh™ at a dose of 2×10^9 PFU/mL and 1×10^9 PFU/mL were not significantly different from one ($P = >0.05$).

12 SUMMARY CONCLUSION OF THE STUDY

- SalmoFresh™ can significantly reduce viable *Salmonella* levels in experimentally contaminated turkey trim by ca. 69-86% in 5 minute contact time, when used at an application rate of ca. 4 mL/lb prior to grinding;
- Using 2-fold dilute SalmoFresh™ preparation (1×10^9 PFU/mL) vs. more concentrated SalmoFresh™ preparation (2×10^9 PFU/mL) does not significantly affect the efficacy.

13 SIGNATURES

(b) (6)

A large rectangular gray box redacting the signature of the first individual.

Manrong Li, MD, M.S.
Research Scientist

(b) (6)

A large rectangular gray box redacting the signature of the second individual.

Alexander Sulakvelidze, Ph.D.
Study Director

Appendix 1.4:
Study #T3BT





T3BT

Efficacy of SalmoFresh[™] (Phage) Application on the Reduction of *Salmonella* spp. in Inoculated Turkey Breast Trim



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Introduction

Salmonella-specific phage, marketed as SalmoFresh™ and produced and distributed by Intralytix, Inc was applied to raw turkey breast trim for this testing. The turkey breast trim was inoculated with a 5-strain cocktail of *Salmonella*, spray-applied with a 1:10 solution of SalmoFresh™ (10X phage concentrate : water), and stuffed into chub casings (~1 lbs each) to determine log reduction values over time at 4°C.

The 1:10 SalmoFresh™ solution was estimated to contain 1×10^9 PFU/mL. PFU = Plaque Forming Units. Intralytix recommended a 1:10 dilution of the concentrate, applied at 2.0 and 4.0 mL per pound of meat. As this was an inoculated study, no organoleptic evaluations were conducted.

Materials and Methods

Salmonella Isolates

Five *Salmonella* isolates were combined (1:1:1:1:1) to form the inoculum in this study. The isolates were *S. Hadar*, *S. Heidelberg*, *S. Agona*, *S. Alachua*, and *S. Schwarzengrund*.

Raw Turkey Meat Block

Raw turkey breast trim pieces were experimentally inoculated and treated with SalmoFresh™ in this study. The moisture, fat, and protein proximate results for the breast trim were 72.1%, 9.5%, and 18.9%, respectively.

Sample Preparation Equipment and Materials

The equipment used in mixing and stuffing of the experimentally inoculated and treated raw turkey meat is commercially available and are a 20 lb capacity stainless steel meat mixer (Lem brand, Harrison, OH) and a 10 lb capacity stainless steel sausage stuffer (Lem brand,

Harrison, OH). Treated meat was stuffed into pre-clipped (on one end) plastic chub casings and sealed with colored cable ties. SalmoFresh™ *Salmonella*-specific phage product was supplied by Intralytix, Inc., Baltimore, MD. A 32 oz. spray bottle (Model # F80HD24; Home Depot) was used for phage application.

Inoculum Preparation

An inoculum containing five strains of *Salmonella* (*S. Hadar*, *S. Heidelberg*, *S. Agona*, *S. Alachua*, and *S. Schwarzengrund*) was aseptically prepared for this study. Each serotype was individually cultured to stationary phase in 250 mL of brain-heart infusion broth (BHI broth; Becton-Dickinson & Co., Sparks, MD) at 37°C for 24 ± 2 hrs. Two-hundred and fifty milliliters of each serotype stationary growth volume was combined into a sterile 2 L Erlenmeyer flask, comprising a total inoculum volume of 1250 mL.

In order to determine the inoculum concentration, serial (1:10) dilutions were performed with 9 mL tubes of Butterfield's buffer (3M, St. Paul, MN). Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter. The inoculum was determined to be 7.90 Log₁₀ CFU/mL.

Meat Block Preparation

Forty pounds (18.1 kg) of breast trim was used as the testing substrate and consisted of un-ground breast trim pieces, that were ground only after inoculation and SalmoFresh™ application in the laboratory (3/16 in. die plate; Model 8-12, Biro Manufacturing Co., Marblehead, OH).

Inoculation of Meat Block

The 40 lb meat block was divided into two, 16 lb (7.26 kg) batches and individually deposited into a 20 lb capacity stainless steel paddle mixer (Lem brand, Harrison, OH). Each 16 lb batch was simultaneously mixed and inoculated with 72.6 mL of the five-strain inoculum ($1:100 = 10^{-2}$); this process was ca. 1 min in duration. Once the batches were inoculated, they were mixed for an additional 3 min; this served as bacterial adhesion time.

SalmoFresh™ Application

SalmoFresh™ *Salmonella*-specific phage (Intralytix, Inc., Baltimore, MD) was acquired for testing. The SalmoFresh™ titer for the sample received was ca. 1×10^{10} PFU/mL, where PFU stands for Plaque Forming Units. Before use, SalmoFresh™ was diluted with sterile, deionized water (1:10.) The diluted titer was ca. 1×10^9 PFU/mL. Two application volumes were used in this study; 2.0 and 4.0 mL/lb of meat. SalmoFresh™ was applied using a 32 oz. spray bottle (Model # F80HD24; Home Depot). Prior to application, the number of required sprays was calculated by counting the number of full sprays into a graduated cylinder that corresponded to the desired volume per lb of treated meat.

Inoculated breast trim was simultaneously mixed and sprayed with 2.0 or 4.0 mL/lb of SalmoFresh™; this process was ca. 1 min in duration. Once the inoculated breast trim was applied with SalmoFresh, it was mixed for an additional 3 min. Prior to stuffing, inoculated, SalmoFresh™-applied breast trim was covered and allowed to sit for 30 min at room temperature to allow for phage attachment. Breast trim was applied with SalmoFresh™ prior to grinding in the laboratory (3/16 in. die plate; Model 8-12, Biro Manufacturing Company, Marblehead, OH).

Stuffing

Inoculated and SalmoFresh™ treated, ground breast trim was stuffed (10 lb capacity stainless steel mixer, Lem brand, Harrison, OH) into pre-clipped plastic chub casings. Target chub weight was 1 lb. Stuffed chubs were closed using cable ties and were held at 4°C until microbiological analysis.

Proximate Analyses

Proximate analyses for moisture, fat, and protein of the un-inoculated, non-SalmoFresh™-treated breast trim was determined by methods approved and described by AOAC International. Moisture, fat, and protein were determined by an NIR method (AOAC 2007.04).

Microbiological Analyses

Pre-SalmoFresh™ Application

Immediately after *Salmonella* inoculation, triplicate, 11 g samples were serially diluted (1:10) with 99 mL of Butterfield's buffer (3M, St. Paul, MN). Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter.

Post-SalmoFresh™ Application

Treated chubs were tested at 24, 48, 72, and 120 hrs post-SalmoFresh™ application for *Salmonella*. For each time period, 3 chubs were aseptically opened and 3 portions (ca. 3.7 g each) from each end and middle of each of the chubs (11 g total per chub), were first serially diluted (1:10) with 99 mL of Butterfield's buffer (10⁻¹) and then with 9 mL Butterfield's buffer (3M, St. Paul, MN), thereafter. Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter.

Statistical Analysis

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; (www.graphpad.com))

Results

When a 1:10 solution of SalmoFresh™ was applied to inoculated breast trim pieces prior to grinding at a volume of 2.0 mL per pound of product, it was effective at reducing *Salmonella* levels by 0.47 log₁₀ CFU/g after 24 hrs of refrigerated storage (Table 1). When the treated breast trim samples were tested after 48 hrs of storage, *Salmonella* reduction increased to 0.68 log₁₀ CFU/g (Table 1). After 72 hrs of storage, *Salmonella* reduction was 0.49 log₁₀ CFU/g; however, after 120 hrs of storage, *Salmonella* was reduced by 0.71 log₁₀ CFU/g (Table 1). Between 24 and 120 hrs of storage, SalmoFresh™ was effectively able to reduce inoculated *Salmonella* between 0.47 and 0.71 log₁₀ CFU/g.

When a 1:10 solution of SalmoFresh™ was applied to inoculated breast trim pieces prior to grinding at an increased volume of 4.0 mL per pound of product, and after 24, 48, 72, and 120 hrs of 4°C storage, it was able to effectively reduce *Salmonella* between 0.63 and 1.02 log₁₀ CFU/g (Table 1). Doubling the application volume of SalmoFresh™ increased the log reduction of *Salmonella* at all time points in the study, but the difference was not statistically significant.

Applying ca. 1x10⁹ PFU/mL SalmoFresh™ to turkey breast trim at the rate of 2.0 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 66%, 79%, 68%, and 80% after ca. 24h, 48h, 72h, and 120h refrigerated (ca. 4°C) storage, respectively. The observed reduction was statistically significant at all time points ($P < 0.001$).

Applying ca. 1×10^9 PFU/mL SalmoFresh[™] to turkey breast trim at the rate of 4.0 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 77%, 91%, 78%, and 83% after ca. 24h, 48h, 72h, and 120h refrigerated (ca. 4°C) storage, respectively. The observed reduction was statistically significant at all time points ($P < 0.001$).

The difference in *Salmonella* recovery among SalmoFresh[™] treated (2 mL/lb) samples at ca. 24h, 48h, 72h, and 120h refrigerated (ca. 4°C) storage was not significant at any of the time points examined ($P > 0.05$).

The difference in *Salmonella* recovery among SalmoFresh[™] treated (4 mL/lb) samples at ca. 24h, 48h, 72h, and 120h refrigerated (ca. 4°C) storage was not significant at any of the time points examined ($P > 0.05$).

The difference in *Salmonella* recovery among SalmoFresh[™] treated (2 mL/lb) and SalmoFresh[™] treated (4 mL/lb) samples was not significant ($P > 0.05$) at all time points.

Conclusion

Treatment with SalmoFresh[™] (2 mL/lb) can significantly reduce *Salmonella* levels in turkey breast trim samples by an average of 73%, but it does not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage).

Treatment with SalmoFresh[™] (4 mL/lb) can significantly reduce *Salmonella* levels in turkey breast trim samples by an average of 82%, but it does not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage).

Using higher application rate (4 mL/lb vs. 2 mL/lb) of SalmoFresh[™] results in numerically better reduction in *Salmonella* levels. However, the differences are not statistically significant ($P > 0.05$).



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Food Scientist

Table 2. One-way analysis of variance (ANOVA) evaluated by comparing the levels of *Salmonella* (CFU/g) recovered from turkey breast trim before and after treatment with a 1:10 SalmoFresh™ phage solution (ca. 1×10^9 PFU/mL) at an application volume of 2.0 or 4.0 mL/lb of product during storage at 4°C.

Comparison	Mean Difference	t	P value
Before treatment vs 2 mL/lb SalmoFresh 24h	458333	6.866	*** P<0.001
Before treatment vs 4 mL/lb SalmoFresh 24h	535000	8.014	*** P<0.001
Before treatment vs 2 mL/lb SalmoFresh 48h	550000	8.239	*** P<0.001
Before treatment vs 4 mL/lb SalmoFresh 48h	630500	9.445	*** P<0.001
Before treatment vs 2 mL/lb SalmoFresh 72h	472500	7.078	*** P<0.001
Before treatment vs 4 mL/lb SalmoFresh 72h	543500	8.142	*** P<0.001
Before treatment vs 2 mL/lb SalmoFresh 120h	559333	8.379	*** P<0.001
Before treatment vs 4 mL/lb SalmoFresh 120h	581333	8.709	*** P<0.001
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (4 mL/lb) 24h	76667	1.148	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (2 mL/lb) 48h	91667	1.373	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (4 mL/lb) 48h	172167	2.579	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (2 mL/lb) 72h	14167	0.2122	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (4 mL/lb) 72h	85167	1.276	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (2 mL/lb) 120h	101000	1.513	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (4 mL/lb) 120h	123000	1.843	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (2 mL/lb) 48h	15000	0.2247	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (4 mL/lb) 48h	95500	1.431	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (2 mL/lb) 72h	-62500	0.9363	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (4 mL/lb) 72h	8500.0	0.1273	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (2 mL/lb) 120h	24333	0.3645	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (4 mL/lb) 120h	46333	0.6941	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (4 mL/lb) 48h	80500	1.206	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (2 mL/lb) 72h	-77500	1.161	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (4 mL/lb) 72h	-6500.0	0.09737	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (2 mL/lb) 120h	9333.3	0.1398	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (4 mL/lb) 120h	31333	0.4694	ns P>0.05
SalmoFresh (4 mL/lb) 48h vs SalmoFresh (2 mL/lb) 72h	-158000	2.367	ns P>0.05
SalmoFresh (4 mL/lb) 48h vs SalmoFresh (4 mL/lb) 72h	-87000	1.303	ns P>0.05
SalmoFresh (4 mL/lb) 48h vs SalmoFresh (2 mL/lb) 120h	-71167	1.066	ns P>0.05
SalmoFresh (4 mL/lb) 48h vs SalmoFresh (4 mL/lb) 120h	-49167	0.7365	ns P>0.05
SalmoFresh (2 mL/lb) 72h vs SalmoFresh (4 mL/lb) 72h	71000	1.064	ns P>0.05
SalmoFresh (2 mL/lb) 72h vs SalmoFresh (2 mL/lb) 120h	86833	1.301	ns P>0.05
SalmoFresh (2 mL/lb) 72h vs SalmoFresh (4 mL/lb) 120h	108833	1.630	ns P>0.05
SalmoFresh (4 mL/lb) 72h vs SalmoFresh (2 mL/lb) 120h	15833	0.2372	ns P>0.05
SalmoFresh (4 mL/lb) 72h vs SalmoFresh (4 mL/lb) 120h	37833	0.5668	ns P>0.05
SalmoFresh (2 mL/lb) 120 vs SalmoFresh (4 mL/lb) 120h	22000	0.3296	ns P>0.05

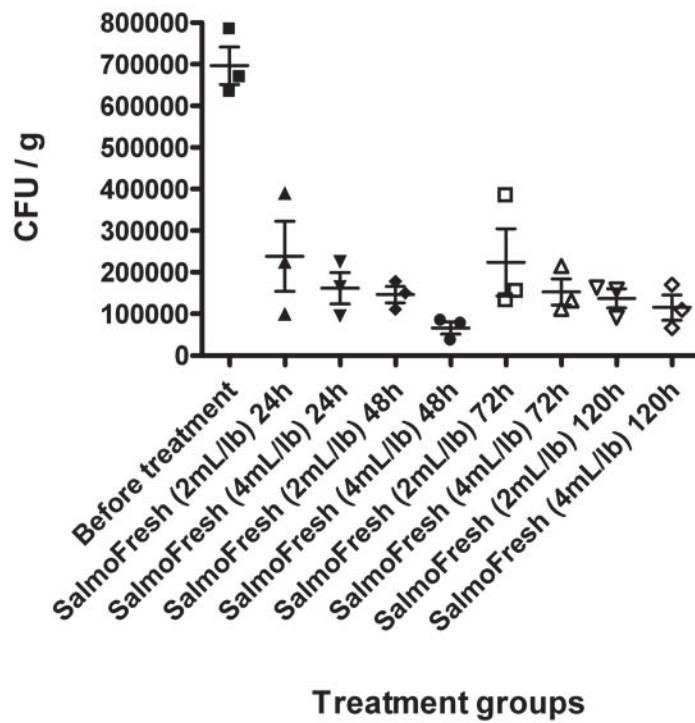


Figure 1 Reduction of *Salmonella* levels in turkey breast trim samples treated with a 1:10 SalmoFresh™ at application volumes of 2.0 mL/lb and 4.0 mL/lb of product during storage at 4°C. Note: Chart was constructed using raw data

Appendix 1.5:
Study #T4DG





T4DG

**Efficacy of SalmoFresh™ (Phage) Application on the
Reduction of *Salmonella* spp. in Inoculated Dark
Ground Turkey Meat**



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Introduction

Salmonella-specific phage, marketed as SalmoFresh™ and produced and distributed by Intralytix, Inc was applied to raw dark ground turkey for this testing. The dark ground turkey was inoculated with a 5-strain cocktail of *Salmonella*, spray-applied with a 1:10 solution of SalmoFresh™ (10X phage concentrate : water), and stuffed into chub casings (~1 lbs each) to determine log reduction values over time at 4°C.

The 1:10 SalmoFresh™ solution was estimated to contain 1×10^9 PFU/mL. PFU = Plaque Forming Units. Intralytix recommended a 1:10 dilution of the concentrate, applied at 2.0 and 4.0 mL per pound of meat. As this was an inoculated study, no organoleptic evaluations were conducted.

Materials and Methods

Salmonella Isolates

Five *Salmonella* isolates were combined (1:1:1:1:1) to form the inoculum in this study. The isolates were *S. Hadar*, *S. Heidelberg*, *S. Agona*, *S. Alachua*, and *S. Schwarzengrund*.

Raw Turkey Meat Block

Raw dark ground turkey was experimentally inoculated and treated with SalmoFresh™ in this study. The moisture, fat, and protein proximate results for the dark ground turkey were 69.9%, 15.4%, and 15.3%, respectively.

Sample Preparation Equipment and Materials

The equipment used in mixing and stuffing of the experimentally inoculated and treated raw turkey meat is commercially available and are a 20 lb capacity stainless steel meat mixer (Lem brand, Harrison, OH) and a 10 lb capacity stainless steel sausage stuffer (Lem brand,

Harrison, OH). Treated meat was stuffed into pre-clipped (on one end) plastic chub casings and sealed with colored cable ties. SalmoFresh[™] *Salmonella*-specific phage product was supplied by Intralytix, Inc., Baltimore, MD. A 32 oz. spray bottle (Model # F80HD24; Home Depot) was used for phage application.

Inoculum Preparation

An inoculum containing five strains of *Salmonella* (*S. Hadar*, *S. Heidelberg*, *S. Agona*, *S. Alachua*, and *S. Schwarzengrund*) was aseptically prepared for this study. Each serotype was individually cultured to stationary phase in 250 mL of brain-heart infusion broth (BHI broth; Becton-Dickinson & Co., Sparks, MD) at 37°C for 24 ± 2 hrs. Two-hundred and fifty milliliters of each serotype stationary growth volume was combined into a sterile 2 L Erlenmeyer flask, comprising a total inoculum volume of 1250 mL.

In order to determine the inoculum concentration, serial (1:10) dilutions were performed with 9 mL tubes of Butterfield's buffer (3M, St. Paul, MN). Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter. The inoculum was determined to be 7.90 Log₁₀ CFU/mL.

Meat Block Preparation

Forty pounds (18.1 kg) of dark ground turkey was acquired from a processing facility.

Inoculation of Meat Block

The 40 lb meat block was divided into two, 16 lb (7.26 kg) batches and individually deposited into a 20 lb capacity stainless steel paddle mixer (Lem brand, Harrison, OH). Each 16 lb batch was simultaneously mixed and inoculated with 72.6 mL of the five-strain inoculum ($1:100 = 10^{-2}$); this process was ca. 1 min in duration. Once the batches were inoculated, they were mixed for an additional 3 min; this served as bacterial adhesion time.

SalmoFreshTM Application

SalmoFreshTM *Salmonella*-specific phage (Intralytix, Inc., Baltimore, MD) was acquired for testing. The SalmoFreshTM titer for the sample received was ca. 1×10^{10} PFU/mL, where PFU stands for Plaque Forming Units. Before use, SalmoFreshTM was diluted with sterile, deionized water (1:10.) The diluted titer was ca. 1×10^9 PFU/mL. Two application volumes were used in this study; 2.0 and 4.0 mL/lb of meat. SalmoFreshTM was applied using a 32 oz. spray bottle (Model # F80HD24; Home Depot). Prior to application, the number of required sprays was calculated by counting the number of full sprays into a graduated cylinder that corresponded to the desired volume per lb of treated meat.

Inoculated dark ground turkey was simultaneously mixed and sprayed with 2.0 or 4.0 mL/lb of SalmoFreshTM; this process was ca. 1 min in duration. Once the inoculated dark ground turkey was applied with SalmoFreshTM, it was mixed for an additional 3 min; this served as bacterial adhesion time. Prior to stuffing, inoculated, SalmoFreshTM-applied dark ground turkey was covered and allowed to sit for 30 min at room temperature to allow for phage attachment.

Stuffing

Inoculated and SalmoFresh™ treated, dark ground turkey was stuffed (10 lb capacity stainless steel mixer, Lem brand, Harrison, OH) into pre-clipped plastic chub casings. Target chub weight was 1 lb. Stuffed chubs were closed using cable ties and were held at 4°C until microbiological analysis.

Proximate Analyses

Proximate analyses for moisture, fat, and protein of the un-inoculated, non-SalmoFresh™-treated dark ground turkey was determined by methods approved and described by AOAC International. Moisture, fat, and protein were determined by an NIR method (AOAC 2007.04).

Microbiological Analyses

Pre-SalmoFresh™ Application

Immediately after *Salmonella* inoculation, triplicate, 11 g samples were serially diluted (1:10) with 99 mL of Butterfield's buffer (3M, St. Paul, MN). Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter.

Post-SalmoFresh™ Application

Treated chubs were tested at 24, 48, 72, and 120 hrs post-SalmoFresh™ application for *Salmonella*. For each time period, 3 chubs were aseptically opened and 3 portions (ca. 3.7 g each) from each end and middle of each of the chubs (11 g total per chub), were first serially diluted (1:10) with 99 mL of Butterfield's buffer (10⁻¹) and then with 9 mL Butterfield's buffer

(3M, St. Paul, MN), thereafter. Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter.

Statistical Analysis

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; (www.graphpad.com))

Results

Two milliliters per pound of a 1:10 solution of SalmoFresh™, when applied to dark ground turkey and stored for 24 hrs resulted in a 0.61 log₁₀ CFU/g reduction in inoculated *Salmonella* (Table 2). After 48, 72, and 120 hrs of refrigerated storage, the 2.0 mL/lb of SalmoFresh™ able to effectively reduce *Salmonella* levels by 0.56, 0.45, and 0.77 log₁₀ CFU/g (Table 2).

Doubling the application volume of SalmoFresh™ increased the log reduction of *Salmonella* in the dark ground turkey chubs. Testing at 24 hrs of storage at this increased application volume revealed the same reduction in *Salmonella* as 120 hrs of storage at 2.0 mL/lb, 0.77 log₁₀ CFU/g (Table 2). Greater log reductions were seen for all time points tested beyond 24 hrs post-application; averaging at 1.03 log₁₀ CFU/g. After 48, 72, and 120 hrs of storage, *Salmonella* reduction in dark ground turkey was 1.12, 0.98, and 1.00 log₁₀ CFU/g (Table 2).

Applying ca. 1x10⁹ PFU/mL SalmoFresh™ to dark ground turkey at the rate of 2.0 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 75%, 73%, 65%, and 83% after ca. 24h, 48h, 72h, and 120h refrigerated (ca. 4°C) storage, respectively. The observed reduction was statistically significant at all time points ($P < 0.001$).

Applying ca. 1×10^9 PFU/mL SalmoFresh[™] to dark ground turkey at the rate of 4.0 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 83%, 92%, 90%, and 90% after ca. 24h, 48h, 72h, and 120h refrigerated (ca. 4°C) storage, respectively. The observed reduction was statistically significant at all time points ($P < 0.001$).

The difference in *Salmonella* recovery among SalmoFresh[™] treated (2 mL/lb) samples at ca. 24h, 48h, 72h, and 120h refrigerated (ca. 4°C) storage were not significant ($P > 0.05$).

The difference in *Salmonella* recovery among SalmoFresh[™] treated (4 mL/lb) samples at ca. 24h, 48h, 72h, and 120h refrigerated (ca. 4°C) storage were not significant ($P > 0.05$).

The difference in *Salmonella* recovery among SalmoFresh[™] treated (2 mL/lb) and SalmoFresh[™] treated (4 mL/lb) samples was not significant ($P > 0.05$) at all time points.

Conclusion

Treatment with SalmoFresh[™] (2 mL/lb) can significantly reduce *Salmonella* levels in dark ground turkey by an average of 74%, but it does not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage).

Treatment with SalmoFresh[™] (4 mL/lb) can significantly reduce *Salmonella* levels in dark ground turkey by an average of 89%, but it does not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage).

Using higher application rate (4 mL/lb vs. 2 mL/lb) of SalmoFresh[™] results in numerically better reduction in *Salmonella* levels in dark ground turkey. However, the differences are not statistically significant ($P > 0.05$).



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Table 1. *Salmonella* spp. plate count (Log₁₀ Mean CFU/g) of dark ground turkey treated with a 1:10 SalmoFresh™ phage solution (ca. 1x10⁹ PFU/mL) at an application volume of 2.0 or 4.0 mL/lb of product during storage at 4°C.

Storage Period	Treatment	Sample #	CFU/g	Mean CFU/g	Mean Log ₁₀ CFU/g	Log Reduction	% Positive	% Reduction
0 hrs	Dark Ground + <i>Salmonella</i>	1	1055000					
		2	1020000	961667	5.98	*	100	*
		3	810000					
24 hrs	Dark Ground + <i>Salmonella</i> + 1:10 Phage (2.0 mL/lb)	1	295000					
		2	235000	236667	5.37	0.61	25	75
		3	180000					
	Dark Ground + <i>Salmonella</i> + 1:10 Phage (4.0 mL/lb)	1	130000					
		2	180000	163333	5.21	0.77	17	83
		3	180000					
48 hrs	Dark Ground + <i>Salmonella</i> + 1:10 Phage (2.0 mL/lb)	1	270000					
		2	285000	264333	5.42	0.56	27	73
		3	238000					
	Dark Ground + <i>Salmonella</i> + 1:10 Phage (4.0 mL/lb)	1	106500					
		2	49500	72333	4.86	1.12	8	92
		3	61000					
72 hrs	Dark Ground + <i>Salmonella</i> + 1:10 Phage (2.0 mL/lb)	1	177500					
		2	235000	337500	5.53	0.45	35	65
		3	600000					
	Dark Ground + <i>Salmonella</i> + 1:10 Phage (4.0 mL/lb)	1	134500					
		2	97000	100000	5.00	0.98	10	90
		3	68500					
120 hrs	Dark Ground + <i>Salmonella</i> + 1:10 Phage (2.0 mL/lb)	1	166000					
		2	218500	164667	5.22	0.77	17	83
		3	109500					
	Dark Ground + <i>Salmonella</i> + 1:10 Phage (4.0 mL/lb)	1	82500					
		2	64000	95833	4.98	1.00	10	90
		3	66500					

Table 2. One-way analysis of variance (ANOVA) evaluated by comparing the levels of *Salmonella* (CFU/g) recovered from dark ground turkey before and after treatment with a 1:10 SalmoFresh™ phage solution (ca. 1×10^9 PFU/mL) at an application volume of 2.0 or 4.0 mL/lb of product during storage at 4°C.

Comparison	Mean Difference	t	P value
Before treatment vs 2 mL/lb SalmoFresh 24h	725000	9.338	*** P<0.001
Before treatment vs 4 mL/lb SalmoFresh 24h	798333	10.283	*** P<0.001
Before treatment vs 2 mL/lb SalmoFresh 48h	697333	8.982	*** P<0.001
Before treatment vs 4 mL/lb SalmoFresh 48h	889333	11.455	*** P<0.001
Before treatment vs 2 mL/lb SalmoFresh 72h	624167	8.039	*** P<0.001
Before treatment vs 4 mL/lb SalmoFresh 72h	861667	11.098	*** P<0.001
Before treatment vs 2 mL/lb SalmoFresh 120h	797000	10.266	*** P<0.001
Before treatment vs 4 mL/lb SalmoFresh 120h	865833	11.152	*** P<0.001
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (4 mL/lb) 24h	73333	0.9446	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (2 mL/lb) 48h	-27667	0.3564	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (4 mL/lb) 48h	164333	2.117	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (2 mL/lb) 72h	-100833	1.299	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (4 mL/lb) 72h	136667	1.760	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (2 mL/lb) 120h	72000	0.9274	ns P>0.05
SalmoFresh (2 mL/lb) 24h vs SalmoFresh (4 mL/lb) 120h	140833	1.814	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (2 mL/lb) 48h	-101000	1.301	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (4 mL/lb) 48h	91000	1.172	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (2 mL/lb) 72h	-174167	2.243	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (4 mL/lb) 72h	63333	0.8157	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (2 mL/lb) 120h	-1333.3	0.01717	ns P>0.05
SalmoFresh (4 mL/lb) 24h vs SalmoFresh (4 mL/lb) 120h	67500	0.8694	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (4 mL/lb) 48h	192000	2.473	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (2 mL/lb) 72h	-73167	0.9424	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (4 mL/lb) 72h	164333	2.117	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (2 mL/lb) 120h	99667	1.284	ns P>0.05
SalmoFresh (2 mL/lb) 48h vs SalmoFresh (4 mL/lb) 120h	168500	2.170	ns P>0.05
SalmoFresh (4 mL/lb) 48h vs SalmoFresh (2 mL/lb) 72h	-265167	3.415	ns P>0.05
SalmoFresh (4 mL/lb) 48h vs SalmoFresh (4 mL/lb) 72h	-27667	0.3564	ns P>0.05
SalmoFresh (4 mL/lb) 48h vs SalmoFresh (2 mL/lb) 120h	-92333	1.189	ns P>0.05
SalmoFresh (4 mL/lb) 48h vs SalmoFresh (4 mL/lb) 120h	-23500	0.3027	ns P>0.05
SalmoFresh (2 mL/lb) 72h vs SalmoFresh (4 mL/lb) 72h	237500	3.059	ns P>0.05
SalmoFresh (2 mL/lb) 72h vs SalmoFresh (2 mL/lb) 120h	172833	2.226	ns P>0.05
SalmoFresh (2 mL/lb) 72h vs SalmoFresh (4 mL/lb) 120h	241667	3.113	ns P>0.05
SalmoFresh (4 mL/lb) 72h vs SalmoFresh (2 mL/lb) 120h	-64667	0.8329	ns P>0.05
SalmoFresh (4 mL/lb) 72h vs SalmoFresh (4 mL/lb) 120h	4166.7	0.05367	ns P>0.05
SalmoFresh (2 mL/lb) 120 vs SalmoFresh (4 mL/lb) 120h	68833	0.8866	ns P>0.05

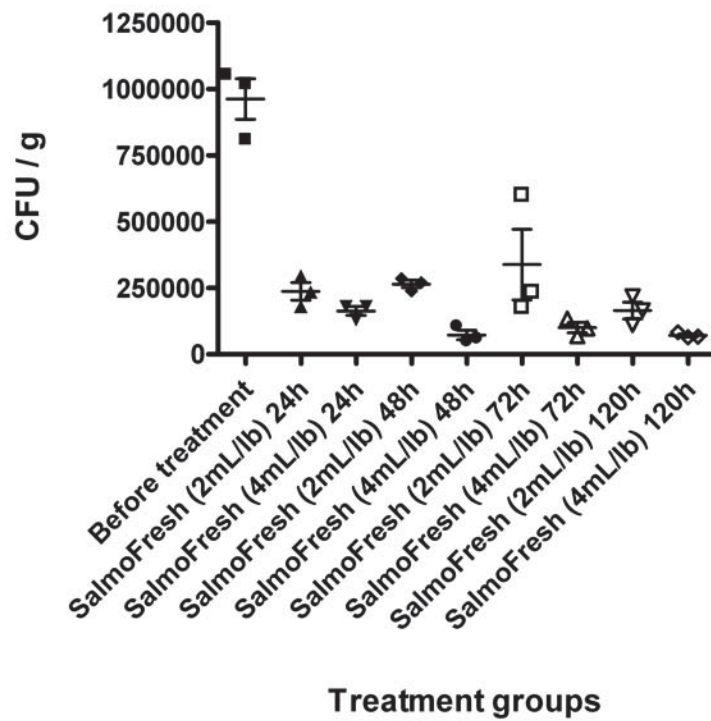


Figure 1 Reduction of *Salmonella* levels in dark ground turkey samples treated with a 1:10 SalmoFresh™ at application volumes of 2.0 mL/lb and 4.0 mL/lb of product during storage at 4°C. Note: Chart was constructed using raw data

Appendix 1.6:
Study #T2DG





T2DG

**Efficacy of SalmoFresh™ (Phage) Application on the
Reduction of *Salmonella* spp. in Inoculated Dark
Ground Turkey Meat**



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Note: Chart was constructed using raw data..... 14

Introduction

Salmonella-specific phage, marketed as SalmoFresh™ and produced and distributed by Intralytix, Inc was applied to raw dark ground turkey for this testing. The dark ground turkey was inoculated with a 5-strain cocktail of *Salmonella*, spray-applied with a 1:10 solution of SalmoFresh™ (10X phage concentrate : water), and stuffed into chub casings (~1 lbs each) to determine log reduction values over time at 4°C.

The 1:10 SalmoFresh™ solution was estimated to contain 1×10^9 PFU/mL. PFU = Plaque Forming Units. Intralytix recommended a 1:10 dilution of the concentrate, applied at 0.5 mL per pound of meat. As this was an inoculated study, no organoleptic evaluations were conducted.

Materials and Methods

Salmonella Isolates

Five *Salmonella* isolates were combined (1:1:1:1:1) to form the inoculum in this study. The isolates were *S. Hadar*, *S. Heidelberg*, *S. Agona*, *S. Alachua*, and *S. Schwarzengrund*.

Raw Turkey Meat Block

Raw dark ground turkey was experimentally inoculated and treated with SalmoFresh™ in this study. The moisture, fat, and protein results were 70.4%, 14.2%, and 15.2%, respectively.

Sample Preparation Equipment and Materials

The equipment used in mixing and stuffing of the experimentally inoculated and treated raw turkey meat is commercially available and are a 20 lb capacity stainless steel meat mixer (Lem brand, Harrison, OH) and a 10 lb capacity stainless steel sausage stuffer (Lem brand,

Harrison, OH). Treated meat was stuffed into pre-clipped (on one end) plastic chub casings and sealed with colored cable ties. SalmoFresh[™] *Salmonella*-specific phage product was supplied by Intralytix, Inc., Baltimore, MD. A 32 oz. spray bottle (Model # F80HD24; Home Depot) was used for phage application.

Inoculum Preparation

An inoculum containing five strains of *Salmonella* (*S. Hadar*, *S. Heidelberg*, *S. Agona*, *S. Alachua*, and *S. Schwarzengrund*) was aseptically prepared for this study. Each serotype was individually cultured to stationary phase in 250 mL of brain-heart infusion broth (BHI broth; Becton-Dickinson & Co., Sparks, MD) at 37°C for 24 ± 2 hrs. Two-hundred and fifty milliliters of each serotype stationary growth volume was combined into a sterile 2 L Erlenmeyer flask, comprising a total inoculum volume of 1250 mL.

In order to determine the inoculum concentration, serial (1:10) dilutions were performed with 9 mL tubes of Butterfield's buffer (3M, St. Paul, MN). Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter. The inoculum was determined to be 8.53 Log₁₀ CFU/mL.

Meat Block Preparation

Sixteen pounds (7.23 kg) of dark ground turkey was acquired from a processing facility for this study.

Inoculation of Meat Block

The 16 lb meat block was deposited into a 20 lb capacity stainless steel paddle mixer (Lem brand, Harrison, OH) and simultaneously mixed and inoculated with 72.3 mL of the five-

strain inoculum ($1:100 = 10^{-2}$); this process was ca. 1 min in duration. Once inoculated, the dark ground turkey was mixed for an additional 3 min; this served as bacterial adhesion time.

SalmoFreshTM Application

SalmoFreshTM *Salmonella*-specific phage (Intralytix, Inc., Baltimore, MD) was acquired for testing. The SalmoFreshTM titer for the sample received was ca. 1×10^{10} PFU/mL, where PFU stands for Plaque Forming Units. Before use, SalmoFreshTM was diluted with sterile, deionized water (1:10.) The diluted titer was ca. 1×10^9 PFU/mL. One application volume was used in this study; 0.5 mL/lb of meat. SalmoFreshTM was applied using a 32 oz. spray bottle (Model # F80HD24; Home Depot). Prior to application, the number of required sprays was calculated by counting the number of full sprays into a graduated cylinder that corresponded to the desired volume per lb of treated meat.

Inoculated dark ground turkey was simultaneously mixed and sprayed with 0.5 mL/lb of SalmoFreshTM; this process was ca. 1 min in duration. Once the inoculated dark ground turkey was applied with SalmoFresh, it was mixed for an additional 3 min. Prior to stuffing, inoculated, SalmoFreshTM-applied dark ground turkey was covered and allowed to sit for 30 min at room temperature to allow for phage attachment.

Stuffing

Inoculated and SalmoFreshTM-treated, dark ground turkey was stuffed (10 lb capacity stainless steel mixer, Lem brand, Harrison, OH) into pre-clipped plastic chub casings. Target chub weight was 1 lb. Stuffed chubs were closed using cable ties and were held at 4°C until microbiological analysis.

Proximate Analyses

Proximate analyses for moisture, fat, and protein of the un-inoculated, non-SalmoFresh™-treated dark ground turkey was determined by methods approved and described by AOAC International. Moisture, fat, and protein were determined by an NIR method (AOAC 2007.04).

Microbiological Analyses

Pre-SalmoFresh™ Application

Immediately after *Salmonella* inoculation, triplicate, 11 g samples were serially diluted (1:10) with 99 mL of Butterfield's buffer (3M, St. Paul, MN). Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter.

Post-SalmoFresh™ Application

Treated chubs were tested at 24, 48, and 96 hrs post-SalmoFresh™ application for *Salmonella*. For each time period, 3 chubs were aseptically opened and 3 portions (ca. 3.7 g each) from each end and middle of each of the chubs (11 g total per chub), were first serially diluted (1:10) with 99 mL of Butterfield's buffer (10^{-1}) and then with 9 mL Butterfield's buffer (3M, St. Paul, MN), thereafter. Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter.

Statistical Analysis

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; (www.graphpad.com))

Results

The results of this study indicate that a 1:10 (0.5mL/lb) SalmoFresh™ solution containing ca. 1×10^9 PFU/mL applied to inoculated dark ground turkey after 24 hours of refrigerated storage, was effective in reducing inoculated *Salmonella* levels by 0.94 log₁₀ CFU/g. Additionally, after 48 and 96 hours of storage, *Salmonella* was reduced by 1.16 and 1.32 log₁₀ CFU/g, respectively (Table 1).

Applying ca. 1×10^9 PFU/mL SalmoFresh™ to dark ground turkey at the rate of 0.5 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 89% after ca. 24 h of incubation at refrigerated temperature (ca. 4°C). The observed reduction was statistically significant ($P < 0.001$).

Applying ca. 1×10^9 PFU/mL SalmoFresh™ to dark ground turkey at the rate of 0.5 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 93% after ca. 48 h of incubation at refrigerated temperature (ca. 4°C). The observed reduction was statistically significant ($P < 0.001$).

Applying ca. 1×10^9 PFU/mL SalmoFresh™ to dark ground turkey at the rate of 0.5 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 95% after ca. 96 h of incubation at refrigerated temperature (ca. 4°C). The observed reduction was statistically significant ($P < 0.001$).

The difference in *Salmonella* recovery among SalmoFresh™ treated dark ground turkey samples at 24 h, 48 h, and 96 h were not significant ($P > 0.05$).

Conclusion

Overall, the application of a 1:10 (0.5mL/lb) solution of SalmoFresh[™], a *Salmonella*-specific phage product to dark ground turkey is able to effectively reduce *Salmonella* spp. by over 1 log₁₀ CFU/g, from 24 to 96 hours post-application, when stored at 4°C.

SalmoFresh[™] significantly (by 89, 93, and 95%) reduced *Salmonella* contamination in dark ground turkey samples stored for 24 h, 48 h, and 96 h; respectively, when applied at the rate of 0.5 mL SalmoFresh[™] (ca. 1x10⁹ PFU/mL) per lb of meat. Reduction in *Salmonella* levels at 24 h, 48 h, and 96 h were similar (differences not statistically significant ($P > 0.05$).)

Treatment with SalmoFresh[™] can significantly reduce *Salmonella* levels in dark ground turkey samples by $\geq 89\%$, but it does not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage).



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Table 1. *Salmonella* spp. plate count (Log₁₀ Mean CFU/g) of dark ground turkey treated with a 1:10 SalmoFresh™ phage solution (ca. 1x10⁹ PFU/mL) at an application volume of 0.5mL/lb of product during storage at 4°C

Storage Period	Treatment	Sample #	CFU/g	Mean CFU/g	Mean Log ₁₀ CFU/g	Log Reduction	% Positive	% Reduction
0 hrs	Dark Ground + <i>Salmonella</i>	1	6900000					
		2	6300000	6600000	6.82	*	100	*
		3	*					
24 hrs	Dark Ground + <i>Salmonella</i> + 1:10 Phage (0.5 mL/lb)	1	745000					
		2	880000	758333	5.88	0.94	11	89
		3	650000					
48 hrs	Dark Ground + <i>Salmonella</i> + 1:10 Phage (0.5 mL/lb)	1	395000					
		2	425000	453333	5.66	1.16	7	93
		3	540000					
96 hrs	Dark Ground + <i>Salmonella</i> + 1:10 Phage (0.5 mL/lb)	1	440000					
		2	305000	315000	5.50	1.32	5	95
		3	200000					

Table 2. One-way analysis of variance (ANOVA) evaluated by comparing the levels of *Salmonella* (CFU/g) recovered from dark ground turkey before and after treatment with a 1:10 SalmoFresh™ phage solution (ca. 1×10^9 PFU/mL) at an application volume of 0.5mL/lb of product during storage at 4°C.

Comparison	Difference	t	P value
Before treatment vs SalmoFresh 24h	5841667	34.040	*** P<0.001
Before treatment vs SalmoFresh 48h	6146667	35.817	*** P<0.001
Before treatment vs SalmoFresh 96h	6285000	36.623	*** P<0.001
SalmoFresh 24h vs SalmoFresh 48h	305000	1.987	ns P>0.05
SalmoFresh 24h vs SalmoFresh 96h	443333	2.888	ns P>0.05
SalmoFresh 48h vs SalmoFresh 96h	138333	0.9012	ns P>0.05

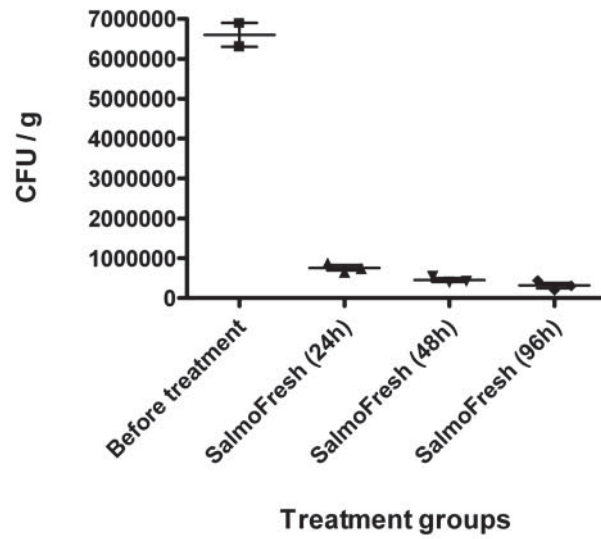


Figure 1 Reduction of *Salmonella* levels in dark ground turkey samples treated with a 1:10 SalmoFresh™ at an application volume of 0.5 mL/lb of product during storage at 4°C.
Note: Chart was constructed using raw data

Appendix 1.7:
Study #T1BT





T1BT

**Efficacy of SalmoFresh[™] (Phage) Application on the
Reduction of *Salmonella* spp. in Inoculated Turkey
Breast Trim**



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Note: Chart was constructed using raw data..... 14

Introduction

Salmonella-specific phage, marketed as SalmoFresh™ and produced and distributed by Intralytix, Inc was applied to raw turkey breast trim for this testing. The turkey breast trim was inoculated with a 5-strain cocktail of *Salmonella*, spray-applied with a 1:10 solution of SalmoFresh™ (10X phage concentrate : water), and stuffed into chub casings (~1 lbs each) to determine log reduction values over time at 4°C.

The 1:10 SalmoFresh™ solution was estimated to contain 1×10^9 PFU/mL. PFU = Plaque Forming Units. Intralytix recommended a 1:10 dilution of the concentrate, applied at 0.5 mL per pound of meat. As this was an inoculated study, no organoleptic evaluations were conducted.

Materials and Methods

Salmonella Isolates

Five *Salmonella* isolates were combined (1:1:1:1:1) to form the inoculum in this study. The isolates were *S. Hadar*, *S. Heidelberg*, *S. Agona*, *S. Alachua*, and *S. Schwarzengrund*.

Raw Turkey Meat Block

Raw turkey breast trim pieces were experimentally inoculated and treated with SalmoFresh™ in this study. The moisture, fat, and protein proximate results were 72.1%, 9.51%, and 18.9%, respectively.

Sample Preparation Equipment and Materials

The equipment used in mixing and stuffing of the experimentally inoculated and treated raw turkey meat is commercially available and are a 20 lb capacity stainless steel meat mixer (Lem brand, Harrison, OH) and a 10 lb capacity stainless steel sausage stuffer (Lem brand,

Harrison, OH). Treated meat was stuffed into pre-clipped (on one end) plastic chub casings and sealed with colored cable ties. SalmoFresh™ *Salmonella*-specific phage product was supplied by Intralytix, Inc., Baltimore, MD. A 32 oz. spray bottle (Model # F80HD24; Home Depot) was used for phage application.

Inoculum Preparation

An inoculum containing five strains of *Salmonella* (*S. Hadar*, *S. Heidelberg*, *S. Agona*, *S. Alachua*, and *S. Schwarzengrund*) was aseptically prepared for this study. Each serotype was individually cultured to stationary phase in 250 mL of brain-heart infusion broth (BHI broth; Becton-Dickinson & Co., Sparks, MD) at 37°C for 24 ± 2 hrs. Two-hundred and fifty milliliters of each serotype stationary growth volume was combined into a sterile 2 L Erlenmeyer flask, comprising a total inoculum volume of 1250 mL.

In order to determine the inoculum concentration, serial (1:10) dilutions were performed with 9 mL tubes of Butterfield's buffer (3M, St. Paul, MN). Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter. The inoculum was determined to be 8.03 Log₁₀ CFU/mL.

Meat Block Preparation

The breast trim was acquired from a processing facility. Sixteen pounds (7.23 kg) of breast trim was used for this meat block and consisted of un-ground breast trim pieces, that were ground only after inoculation and SalmoFresh™ application in the laboratory (3/16 in. die plate; Model 8-12, Biro Manufacturing Company, Marblehead, OH).

Inoculation of Meat Block

The 16 lb meat block was deposited into a 20 lb capacity stainless steel paddle mixer (Lem brand, Harrison, OH) and simultaneously mixed and inoculated with 72.3 mL of the five-strain inoculum ($1:100 = 10^{-2}$); this process was ca. 1 min in duration. Once inoculated, the trim was mixed for an additional 3 min; this served as bacterial adhesion time.

SalmoFreshTM Application

SalmoFreshTM *Salmonella*-specific phage (Intralytix, Inc., Baltimore, MD) was acquired for testing. The SalmoFreshTM titer for the sample received was ca. 1×10^{10} PFU/mL, where PFU stands for Plaque Forming Units. Before use, SalmoFreshTM was diluted with sterile, deionized water (1:10.). The diluted titer was ca. 1×10^9 PFU/mL. One application volume was used in this study; 0.5 mL/lb of meat. SalmoFreshTM was applied using a 32 oz. spray bottle (Model # F80HD24; Home Depot). Prior to application, the number of required sprays was calculated by counting the number of full sprays into a graduated cylinder that corresponded to the desired volume per lb of treated meat.

Inoculated breast trim was simultaneously mixed and sprayed with 0.5 mL/lb of SalmoFreshTM; this process was ca. 1 min in duration. Once the inoculated breast trim was applied with SalmoFresh, it was mixed for an additional 3 min. Prior to stuffing, inoculated, SalmoFreshTM-applied breast trim was covered and allowed to sit for 30 min at room temperature to allow for phage attachment. Breast trim was applied with SalmoFreshTM prior to grinding in the laboratory (3/16 in. die plate; Model 8-12, Biro Manufacturing Company, Marblehead, OH).

Stuffing

Inoculated and SalmoFresh™ treated, ground breast trim was stuffed (10 lb capacity stainless steel mixer, Lem brand, Harrison, OH) into pre-clipped plastic chub casings. Target chub weight was 1 lb. Stuffed chubs were closed using cable ties and were held at 4°C until microbiological analysis.

Proximate Analyses

Proximate analyses for moisture, fat, and protein of the un-inoculated, non-SalmoFresh™-treated breast trim was determined by methods approved and described by AOAC International. Moisture, fat, and protein were determined by an NIR method (AOAC 2007.04).

Microbiological Analyses

Pre-SalmoFresh™ Application

Immediately after *Salmonella* inoculation, triplicate, 11 g samples were serially diluted (1:10) with 99 mL of Butterfield's buffer (3M, St. Paul, MN). Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter.

Post-SalmoFresh™ Application

Treated chubs were tested at 24 and 96 hrs post-SalmoFresh™ application for *Salmonella*. For each time period, 3 chubs were aseptically opened and 3 portions (ca. 3.7 g each) from each end and middle of each of the chubs (11 g total per chub), were first serially diluted (1:10) with 99 mL of Butterfield's buffer (10⁻¹) and then with 9 mL Butterfield's buffer (3M, St. Paul, MN), thereafter. Each (1:10) dilution was spread-plated onto XLT4 agar (Becton-Dickinson & Co., Sparks, MD) incubated at 37°C for 24 ± 2 hrs, and enumerated thereafter.

Statistical Analysis

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; (www.graphpad.com))

Results

The results of this study indicate that a 1:10 (0.5mL/lb) SalmoFresh™ solution containing ca. 1×10^9 PFU/mL applied to inoculated turkey breast trim prior to grinding and after 24 hours of refrigerated storage, reduced *Salmonella* levels 0.59 log₁₀ CFU/g and 0.67 log₁₀ CFU/g after 96 hrs (Table 1).

Applying ca. 1×10^9 PFU/mL SalmoFresh™ to turkey breast trim at the rate of 0.5 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 74% after ca. 24 h of incubation at refrigerated temperature (ca. 4°C). The observed reduction was statistically significant ($P < 0.05$). Applying ca. 1×10^9 PFU/mL SalmoFresh™ to turkey breast trim at the rate of 0.5 mL per lb of turkey reduced the number of viable *Salmonella* by ca. 79% after ca. 96 h of incubation at refrigerated temperature (ca. 4°C). The observed reduction was statistically significant ($P < 0.05$). The difference in *Salmonella* recovery between SalmoFresh™ treated samples at 24 h and 96 h were not significant ($P > 0.05$).

Conclusion

This data indicates that when SalmoFresh[™] is applied to turkey breast trim pieces prior to grinding, it is able to effectively reduce *Salmonella* spp. 0.67 log₁₀ CFU/g up to 96 hrs post-application, when stored at 4°C.

At the application and storage conditions used during this study, SalmoFresh[™] significantly (by 74% and 79%) reduced *Salmonella* contamination in turkey breast trim samples stored for 24 h and 96 h, respectively, when applied at the rate of 0.5 mL SalmoFresh[™] (ca. 1x10⁹ PFU/mL) per lb of turkey meat. Reduction in *Salmonella* levels at 24 h and 96 h was similar (differences not statistically significant ($P > 0.05$).)

Treatment with SalmoFresh[™] can significantly reduce *Salmonella* levels in turkey breast trim samples by $\geq 74\%$, but it does not provide continued technical effect (i.e., effect is limited to the initial reduction and does not improve during storage).



January 10, 2012

Food Scientist

Table 1. *Salmonella* spp. plate count (Log₁₀ Mean CFU/g) of turkey breast trim treated with a 1:10 SalmoFresh™ phage solution (ca. 1x10⁹ PFU/mL) at an application volume of 0.5mL/lb of product during storage at 4°C

Storage Period	Treatment	Sample #	CFU/g	Mean CFU/g	Mean Log ₁₀ CFU/g	Log Reduction	% Positive	% Reduction
0 hrs	Breast Trim + <i>Salmonella</i>	1	525000	736667	5.87	*	100	*
		2	1035000					
		3	650000					
24 hrs	Breast Trim + <i>Salmonella</i> + 1:10 Phage (0.5 mL/lb)	1	143333	191111	5.28	0.59	26	74
		2	150000					
		3	280000					
96 hrs	Breast Trim + <i>Salmonella</i> + 1:10 Phage (0.5 mL/lb)	1	122667	156611	5.19	0.67	21	79
		2	103500					
		3	243667					

Table 2. One-way analysis of variance (ANOVA) evaluated by comparing the levels of *Salmonella* (CFU/g) recovered from turkey breast trim before and after treatment with a 1:10 SalmoFresh™ phage solution (ca. 1x10⁹ PFU/mL) at an application volume of 0.5mL/lb of product during storage at 4°C.

Comparison	Difference	t	P value
Before treatment vs. SalmoFresh 24h	545556	5.703	* P<0.05
Before treatment vs. SalmoFresh 96h	580055	6.063	* P<0.05
SalmoFresh 24h vs. SalmoFresh 96h	34500	0.3606	ns P>0.05

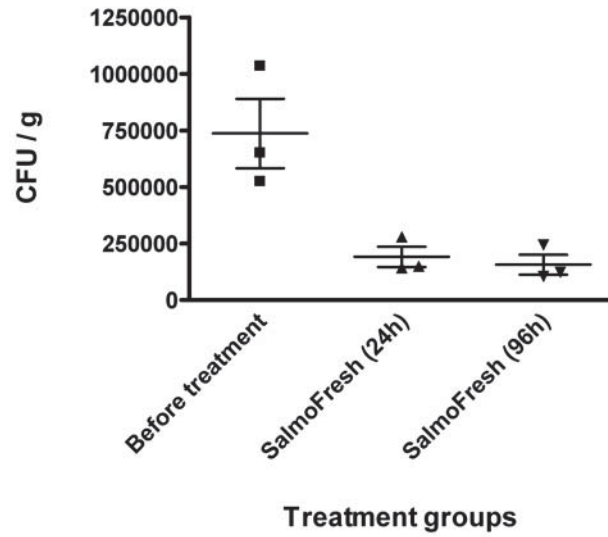


Figure 1 Reduction of *Salmonella* levels in turkey breast trim samples treated with a 1:10 SalmoFresh™ at an application volume of 0.5 mL/lb of product during storage at 4°C. Note: Chart was constructed using raw data

Appendix 1.8:
Study #SS11L19ML





**Evaluation of the ability of SalmoFresh to
reduce *Salmonella* contamination in
experimentally contaminated turkey trim when
applied at the rate of 4.0 mL per lb of poultry
prior to grinding.**

Study # SS11L19ML

Intralaytix
The Columbus Center
701 E. Pratt St.
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1 STUDY TITLE

Evaluation of the ability of SalmoFresh™ to reduce *Salmonella* contamination in experimentally contaminated turkey trim when applied at the rate of 4.0 mL per lb of poultry prior to grinding.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D	Chief Scientist	Study Director
Manrong Li, M.S.	Research Scientist	Hands-on-research

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of SalmoFresh™ reduces the number of viable *Salmonella* in ground turkey when applied at the rate of 4mL per lb of poultry prior to grinding.

6 TEST MATRIX

A sample of turkey trim was obtained from [REDACTED]. It was not washed or pre-treated prior to our studies.

7 SALMOFRESH LOT AND APPLICATION RATE

- SalmoFresh™ Lot #02TestSample
- Titer: approx. 1×10^9 PFU/mL
- SalmoFresh™ was applied using Basic Spray Gun Model #250-2 (Badger Air-Brush Co., Franklin Park, IL).
- The application rate was ca. 4mL SalmoFresh™ per 1 pound of poultry.

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE POULTRY

The poultry test matrix was experimentally contaminated with a single *Salmonella* strains:

- *S.He902*: A nalidixic acid resistant mutant developed from *S.He899* (also known as ATCC8326, *Salmonella enterica* serotype Heidelberg.)

The strain was selected for nalidixic acid resistance by serially passaging the original isolate on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strain underwent ≤ 8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 $\mu\text{g/ml}$. After the passaging, the above-noted Intralytix strain designation was assigned (i.e., *S.He902*). The strain was stored at -80°C , at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 μg of nalidixic acid/ml.

Shortly before performing the study, the strain was thawed and grown ($37 \pm 2^{\circ}\text{C}$, 24-48 h) in NZCYM broth supplemented with nalidixic acid (25 $\mu\text{g/ml}$) until the culture reached an OD_{600} of ca. 1.5, which corresponds to ca. 1×10^9 CFU/mL. The bacterial culture was diluted 1000-fold just prior to performing the study.

The turkey was experimentally contaminated by ca. 1,250 CFU of the above-defined *Salmonella* culture / g of turkey trim.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Salmonella/Shigella Agar (SSA) (Becton, Dickinson and Co., Sparks, MD; cat #274500)

10 GENERAL OUTLINE OF STUDY

- 1) The challenge dose of bacteria was applied onto the matrix samples' surfaces. Bacterial cultures were evenly spread onto all sides of the poultry sample surfaces using hockey sticks. One sample was not treated with bacterial cultures as the uncontaminated, untreated control.
- 2) The bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
- 3) Water (control) or SalmoFresh™ was applied as described in section 7. Poultry samples were rotated and all sides of the samples were sprayed, to ensure reasonably even coverage of the entire surface.
- 4) The samples were covered and incubated at room temperature for ca. 5 minutes.
- 5) At 5 minutes post-treatment with water or SalmoFresh™, all samples (including uncontaminated, untreated control) were ground using a #10 meat grinder (Kitchener #508313).
- 6) Each sample was covered and stored at 4°C for 24 ± 2 hr.

- 7) At 24 hr, from each sample group, triplicate ~25g samples of ground meat were removed, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds.
- 8) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.3 mL) of the stomached meat/peptone water mixture onto separate SSA plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\text{Total CFU/g of treated poultry} = \text{CFU} / \text{ml plated} \times \text{ml peptone water} / \text{g sample analyzed}$$

- 9) All of the remaining samples (from step 7)) were re-contaminated as described in step 1).
- 10) Each sample was covered and stored at 4°C for 24±2 hr.
- 11) At 24 hr, from each sample group, triplicate ~25g samples of ground meat were removed, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds.
- 12) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.3 mL) of the stomached meat/peptone water mixture onto separate SSA plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\text{Total CFU/g of treated poultry} = \text{CFU} / 0.3 \text{ mL plated} \times 225 \text{ ml peptone water} / 25 \text{ g sample analyzed}$$

Counts from 0.3 mL plating were used during all analyses, because they provided most robust, countable numbers (i.e., more than 10 whenever possible but less than 100 colonies per plate)

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #SS11L19ML

Sample	Bacteria	Treatment	Treatment			Recontamination		
			25g replicate samples	CFU in 0.3 ml	CFU/g	Bacteria	CFU in 0.3 ml	CFU/g
A	Yes	SalmoFresh™	3	3; 8; 7	90; 240; 210	Yes	23; 19; 27	690; 570; 810
B	Yes	Water	3	20; 22; 21	600; 660; 630	Yes	85; 79; 45	2550; 2370; 1350
C	No	None	3	0; 0; 0	0; 0; 0	Yes	26; 40; 65	780; 1200; 1950

11.2 Tabular presentation of results

Table 2 Reduction of *Salmonella* counts in ground turkey treated with ca. 1×10^9 PFU/mL SalmoFresh when applied at the rate of 4mL per lb of poultry prior to grinding.

Challenged with <i>Salmonella</i>	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Significant?
Yes	1×10^9 PFU/mL SalmoFresh™	$n = 3$	180	71%	Yes
Yes	Water	$n = 3$	630		

Table 3 Effect of original treatments upon recovery of *Salmonella* applied 24 hours post-treatment (re-contamination.)

Original Treatment	Initial Mean CFU/g	Challenged with additional <i>Salmonella</i>	Replicates	Mean CFU/g	Significant?
1×10^9 PFU/mL SalmoFresh™	180	Yes	$n = 3$	690	No
Untreated	0	Yes	$n = 3$	1310	

11.3 Graphical presentation of efficacy of results

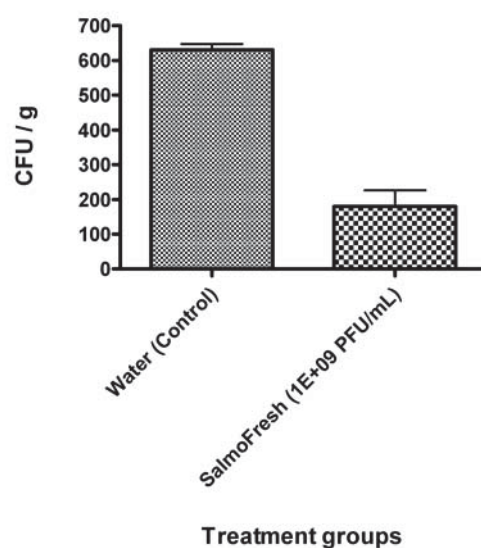


Figure 1 Reduction of *Salmonella* counts in ground turkey treated with ca. 1×10^9 PFU/mL SalmoFresh when applied at the rate of 4mL per lb of poultry prior to grinding. (mean and standard error)

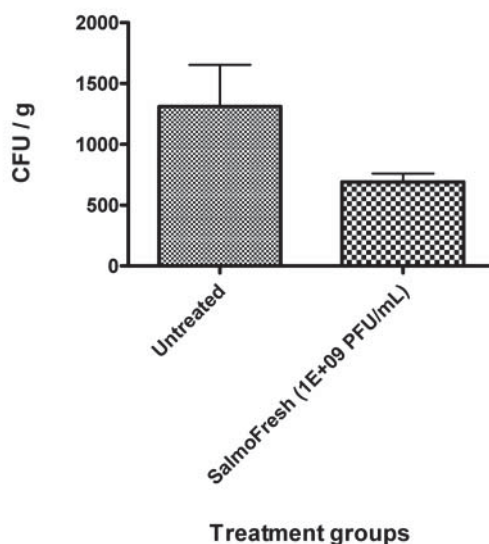


Figure 2 Recovery of *Salmonella* from SalmoFresh[™]-treated and untreated turkey meat samples after recontamination (mean and standard error)

11.4 Statistical analysis

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; www.graphpad.com)

The efficacy of the SalmoFresh[™] treatment in reducing the number of viable *Salmonella* in the experimentally contaminated ground turkey samples was evaluated by comparing the data obtained with the water-treated control samples and the SalmoFresh[™]-treated samples (Figure 1). Statistical analysis employed: unpaired t test.

Unpaired t test

Do the means of SalmoFresh[™] and Water differ significantly?

P value

The two-tailed P value is 0.0008, considered extremely significant.

The impact of recontamination on the efficacy of SalmoFresh[™] (i.e., whether or not original SalmoFresh[™] treatment provided residual technical effect / continued protection against recontamination with *Salmonella*) was evaluated by re-contaminating samples with *Salmonella*, and comparing CFU/g data between (i) SalmoFresh[™]-treated turkey samples (re-contaminated), and (ii) untreated turkey samples contaminated with the same challenge dose of *Salmonella* (Figure 2). Statistical analysis employed: unpaired t test.

Unpaired t test

Do the means of SalmoFresh (re-contaminated) and No treatment (re-contaminated) differ significantly?

The two-tailed P value is 0.1504, considered not significant.

11.5 Brief discussion of results and study's conclusions

- Applying 1×10^9 PFU/mL SalmoFresh™ to turkey trim prior to grinding – at the rate of 4.0 mL per lb of poultry - reduced the number of viable *Salmonella* by ca. 71% after ca. 24 hours of incubation at 4°C. The observed reduction was statistically significant ($P = < 0.05$).
- When SalmoFresh™- treated turkey samples were re-contaminated with *Salmonella*, and the same dose of *Salmonella* was applied onto uncontaminated and untreated turkey meat, the difference in *Salmonella* recovery was ca. 47% between the two groups, respectively. The difference was statistically not significant ($P = > 0.05$). Thus, SalmoFresh™-treatment did not significantly protect the ground turkey meat from subsequent recontamination with *Salmonella* (i.e., SalmoFresh™ provided no continued technical effect).

12 SUMMARY CONCLUSION OF THE STUDY

SalmoFresh™ significantly reduced *Salmonella* levels in turkey trim samples by ca. 71% when it was applied to the experimentally contaminated meat before grinding. However, SalmoFresh™ treatment did not have a residual protective effect in the ground meat; i.e., it did not significantly protect the ground turkey meat from subsequent recontamination with *Salmonella*.

Appendix 1.9 :
Study #SS11L26ML





**Evaluation of the ability of SalmoFresh to
reduce *Salmonella* contamination in
experimentally contaminated chicken breast
when applied at the rate of 4.0 mL per lb of
poultry prior to grinding.**

Study # SS11L26ML

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1 STUDY TITLE

Evaluation of the ability of SalmoFresh™ to reduce *Salmonella* contamination in experimentally contaminated chicken breast when applied at the rate of 4.0 mL per lb of poultry prior to grinding.

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D	Chief Scientist	Study Director
Manrong Li, M.S.	Research Scientist	Hands-on-research

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of SalmoFresh™ reduces the number of viable *Salmonella* in ground chicken when applied at the rate of 4mL per lb of poultry prior to grinding.

6 TEST MATRIX

A sample of chicken breast was purchased at a Baltimore area supermarket. It was not washed or pre-treated prior to our studies.

7 SALMOFRESH LOT AND APPLICATION RATE

- SalmoFresh™ Lot #02TestSample
- Titer: approx. 1×10^9 PFU/mL
- SalmoFresh™ was applied using Basic Spray Gun Model #250-2 (Badger Air-Brush Co., Franklin Park, IL).
- The application rate was ca. 4mL SalmoFresh™ per 1 pound of poultry.

8 BACTERIAL STRAINS USED TO EXPERIMENTALLY CONTAMINATE POULTRY

The poultry test matrix was experimentally contaminated with a single *Salmonella* strains:

- *S.E900*: A nalidixic acid resistant mutant developed from *S.E660* (also known as ATCC13076, *Salmonella enterica* serotype Enteritidis.)
- *S.Ty901*: A nalidixic acid resistant mutant developed from *S.Ty653* (also known as ATCC6539, *Salmonella enterica* serotype Typhi.)
- *S.He902*: A nalidixic acid resistant mutant developed from *S.He899* (also known as ATCC8326, *Salmonella enterica* serotype Heidelberg.)

The strains were selected for nalidixic acid resistance by serially passaging the original isolates on LB agar plates supplemented with increasing concentrations of nalidixic acid. Each strain underwent ≤ 8 serial passages before it was determined to be nalidixic acid-resistant at a concentration of 25 $\mu\text{g/ml}$. After the passaging, the above-noted Intralytix strain designations were assigned (i.e., *S.E900*, *S.Ty901*, and *S.He902*). The strains were stored at -80°C , at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 μg of nalidixic acid/ml.

Shortly before performing the study, the three strains were thawed and grown ($37 \pm 2^{\circ}\text{C}$, 24-48 h) in LB broth supplemented with nalidixic acid (25 $\mu\text{g/ml}$) until the cultures reached an OD_{600} of ca. 1.5, which corresponds to ca. 1×10^9 CFU/mL. Equal volumes of three bacterial cultures were mixed and the mixture diluted 1000-fold just prior to performing the study.

The chicken was experimentally contaminated by ca. 750 CFU of the above-defined *Salmonella* culture / g of chicken breast.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Salmonella/Shigella Agar (SSA) (Becton, Dickinson and Co., Sparks, MD; cat #274500)

10 GENERAL OUTLINE OF STUDY

- 1) The challenge dose of bacteria was applied onto the matrix samples' surfaces. Bacterial cultures were evenly spread onto all sides of the poultry sample surfaces using hockey sticks. One sample was not treated with bacterial cultures as the uncontaminated, untreated control.
- 2) The bacteria were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
- 3) Water (control) or SalmoFresh™ was applied as described in section 7. Poultry samples were rotated and all sides of the samples were sprayed, to ensure reasonably even coverage of the entire surface.
- 4) The samples were covered and incubated at room temperature for ca. 5 minutes.

- 5) At 5 minutes post-treatment with water or SalmoFresh™, all samples (including uncontaminated, untreated control) were ground using a #10 meat grinder (Kitchener #508313).
- 6) Each sample was covered and stored at 4°C for 24±2 hr.
- 7) At 24 hr, from each sample group, triplicate ~25g samples of ground meat were removed, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds.
- 8) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.3 mL) of the stomached meat/peptone water mixture onto separate SSA plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\text{Total CFU/g of treated poultry} = \text{CFU} / \text{ml plated} \times \text{ml peptone water} / \text{g sample analyzed}$$

- 9) All of the remaining samples (from step 7)) were recontaminated as described in step 1).
- 10) Each sample was covered and stored at 4°C for 24±2 hr.
- 11) At 24 hr, from each sample group, triplicate ~25g samples of ground meat were removed, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds.
- 12) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.3 mL) of the stomached meat/peptone water mixture onto separate SSA plates supplemented with nalidixic acid (25 mg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\text{Total CFU/g of treated poultry} = \text{CFU} / \text{ml plated} \times \text{ml peptone water} / \text{g sample analyzed}$$

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #SS11L26ML

Sample	Bacteria	Treatment	Treatment			Recontamination		
			25g replicate samples	CFU in 0.3 ml	CFU/g	Bacteria	CFU in 0.3 ml	CFU/g
A	Yes	SalmoFresh™	3	3; 3; 8	90; 90; 240	Yes	18; 24; 28	540; 720; 840
B	Yes	Water	3	17; 12; 16	510; 360; 480	Yes	34; 31; 32	1020; 930; 960
C	No	None	3	0; 0; 0	0; 0; 0	Yes	29; 28; 18	870; 840; 540

11.2 Tabular presentation of results

Table 2 Reduction of *Salmonella* counts in ground chicken treated with ca. 1×10^9 PFU/mL SalmoFresh™ when applied at the rate of 4mL per lb of poultry prior to grinding.

Challenged with <i>Salmonella</i>	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Significant?
Yes	1×10^9 PFU/mL SalmoFresh™	$n = 3$	140	69%	Yes
Yes	Water	$n = 3$	450		

Table 3 Effect of original treatments upon recovery of *Salmonella* applied 24 hours post-treatment.

Original Treatment	Initial Mean CFU/g	Challenged with additional <i>Salmonella</i>	Replicates	Mean CFU/g	Significant?
1×10^9 PFU/mL SalmoFresh™	140	Yes	$n = 3$	700	No
Water	450	Yes	$n = 3$	970	No
Untreated	0	Yes	$n = 3$	750	

11.3 Graphical presentation of efficacy of results

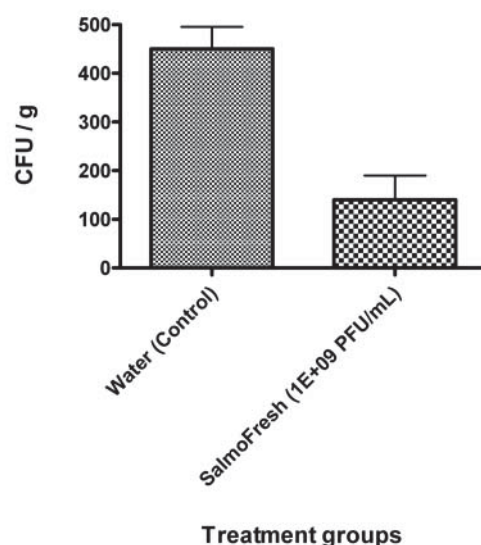


Figure 1 Reduction of *Salmonella* counts in ground chicken treated with ca. 1×10^9 PFU/mL SalmoFresh™ when applied at the rate of 4mL per lb of poultry prior to grinding (mean and standard error.)

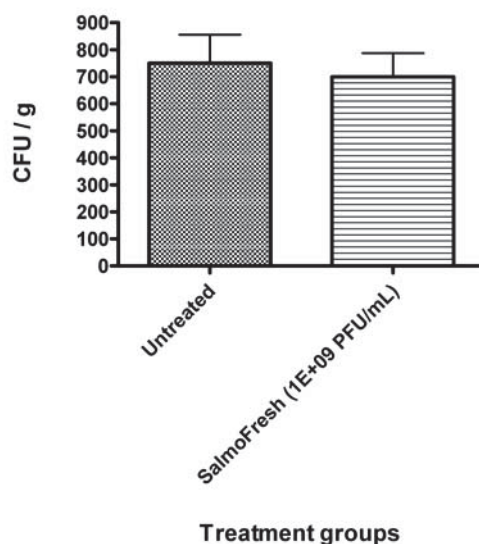


Figure 2 Recovery of *Salmonella* from SalmoFresh[™]-treated and untreated chicken meat samples after recontamination (mean and standard error.)

11.4 Statistical analysis

Statistical analysis was performed using version 3.1a of GraphPad InStat for Macintosh and version 5.0d GraphPad Prism for Macintosh (GraphPad Software; San Diego, CA; www.graphpad.com)

The efficacy of the SalmoFresh[™] treatment in reducing the number of viable *Salmonella* in the experimentally contaminated ground chicken samples was evaluated by comparing the data obtained with the water-treated control samples and the SalmoFresh[™]-treated samples (Figure 1). Statistical analysis employed: unpaired t test.

Unpaired t test

Do the means of SalmoFresh[™] and Water differ significantly?

P value

The two-tailed P value is 0.0103, considered significant.

The impact of recontamination on the efficacy of SalmoFresh[™] (i.e., whether or not original SalmoFresh[™] treatment provided residual technical effect / continued protection against recontamination with *Salmonella*) was evaluated by re-contaminating samples with *Salmonella*, and comparing CFU/g data between (i) SalmoFresh[™]-treated chicken samples (re-contaminated), and (ii) untreated chicken samples contaminated with the same challenge dose of *Salmonella* (Figure 2). Statistical analysis employed: unpaired t test.

Unpaired t test

Do the means of SalmoFresh (re-contaminated) and Untreated (re-contaminated) differ significantly?

The two-tailed P value is 0.7332, considered not significant.

11.5 Brief discussion of results and study's conclusions

- Applying 1×10^9 PFU/mL SalmoFresh™ to chicken breast prior to grinding – at the rate of 4.0 mL per lb of poultry - reduced the number of viable *Salmonella* by ca. 69% after ca. 24 hours of incubation at 4°C. The observed reduction was statistically significant.
- When SalmoFresh™- treated chicken samples were re-contaminated with *Salmonella*, and the same dose of *Salmonella* was applied onto uncontaminated and untreated chicken meat, the difference in *Salmonella* recovery was ca. 6.7% between the two groups, respectively. The difference was statistically not significant ($P = > 0.05$). Thus, SalmoFresh™-treatment did not significantly protect the ground chicken meat from subsequent recontamination with *Salmonella* (i.e., SalmoFresh™ provided no continued technical effect).

12 SUMMARY CONCLUSION OF THE STUDY

SalmoFresh™ significantly reduced *Salmonella* levels in chicken trim samples by ca. 69% when it was applied to the experimentally contaminated meat before grinding. However, SalmoFresh™ treatment did not have a residual protective effect in the ground meat; i.e., it did not significantly protect the ground chicken meat from subsequent recontamination with *Salmonella*.

PR



Ramos-Valle, Moraima

From: Alexander Sulakvelidze [asulakvelidze@intralytix.com]
Sent: Thursday, July 05, 2012 1:25 PM
To: Ramos-Valle, Moraima
Cc: Joelle Woolston
Subject: Re: Sections marked "Confidential" in Intralytix's GRAS Notification for SalmoFresh
Attachments: FDA Letter _ July 5 2012.pdf

Dear Dr. Moraima Ramos-Valle,

Please find attached letter, per our phone conversation today.

I would appreciate a brief acknowledgment that you received this e-mail.

Sincerely,

Sandro Sulakvelidze

Alexander Sulakvelidze, Ph.D.
Vice President, Research & Development
Chief Scientist
Intralytix, Inc.
The Columbus Center
701 E. Pratt Street
Baltimore, MD 21202

Phone: 410-625-2533
Fax: 410-625-2506
E-mail: asulakvelidze@intralytix.com

www.intralytix.com

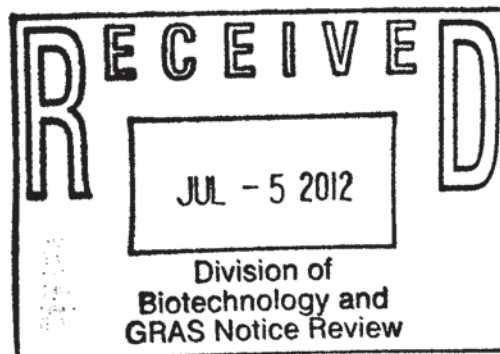
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July 5, 2012

Dr. Moraima Ramos-Valle
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
U.S. Food & Drug Administration
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740



Re: Sections marked "Confidential" in Intralytix's GRAS Notification for SalmoFresh™

Dear Dr. Ramos-Valle:

I am writing this letter as a follow up to our telephone conversation on July 5, 2012 with regards to the above-referenced matter. As you correctly stated during that phone conversation, we have marked "confidential" some of the efficacy data performed by our industry collaborator. These data were contained in Appendices 1.1 – 1.9 of the GRAS package we submitted to the FDA on June 25, 2012. The purpose of this letter is to clarify that it is only the name of that industry partner that we wish to keep confidential. Everything else in the package is non-confidential. I hope this clarifies the issue. Please let me know if you have any additional questions.

Sincerely,

(b) (6)

Alexander Sulakvelidze, Ph.D.
Vice President
Chief Scientist

000155

SUBMISSION END

000156



**Evaluation of the ability of SalmoFresh to
reduce *Salmonella* contamination in
experimentally contaminated cantaloupe**

Study # SF12G16ML1

Intralytix

The Columbus Center

701 E. Pratt St.

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1 STUDY TITLE

Evaluation of the ability of SalmoFresh™ to reduce *Salmonella* contamination in experimentally contaminated cantaloupe

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Manrong Li, MD	Sr. Research Scientist	Hands-on-research

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
701 E. Pratt St.
Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of SalmoFresh™ reduces the number of viable *Salmonella* on cantaloupe when applied at the rate of 4mL per lb or 2mL per lb.

6 TEST MATRIX

Pre-cut cantaloupe chunks were obtained from a local Baltimore supermarket. They were not washed or pre-treated prior to our studies.

7 SALMOFRESH LOT AND APPLICATION RATE

- SalmoFresh™ Lot# 0211C150168
- Titer: approx. 1×10^9 PFU/mL
- SalmoFresh™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).
- The application rate was ca. 4mL SalmoFresh™ per lb or 2mL SalmoFresh™ per lb cantaloupe.

8 SALMONELLA STRAINS USED TO EXPERIMENTALLY CONTAMINATE CANTALOUPE

The cantaloupe test matrix was experimentally contaminated with *Salmonella* strains:

- *S.E900*: A nalidixic acid resistant mutant developed from *S.E660* (also known as ATCC13076, *Salmonella enterica* serotype Enteritidis.)
- *S.Ty901*: A nalidixic acid resistant mutant developed from *S.Ty653* (also known as ATCC6539, *Salmonella enterica* serotype Typhi.)
- *S.He902*: A nalidixic acid resistant mutant developed from *S.He899* (also known as ATCC8326, *Salmonella enterica* serotype Heidelberg.)

The strains were selected for nalidixic acid resistance by serially passaging the original isolates on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strains underwent ≤ 8 serial passages before they were determined to be nalidixic acid-resistant at a concentration of 25 $\mu\text{g/mL}$. After the passaging, the above-noted Intralytix strain designations were assigned (i.e., *S.E900*, *S.Ty901*, *S.He902*). The strains were stored at -80°C , at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 μg of nalidixic acid/mL.

Shortly before performing the study, the strains were thawed and grown ($37 \pm 2^\circ\text{C}$, 16-24 h) in LB broth supplemented with nalidixic acid (25 $\mu\text{g/mL}$). Overnight growth corresponds to ca. 4×10^8 CFU/mL. The cultures were mixed in equal parts and the mixture was diluted 1000-fold just prior to performing the study.

The cantaloupe were experimentally contaminated by ca. 2000 CFU of the above-defined 1:1:1 mixture of three *Salmonella* strains / g of cantaloupe.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Hektoen-Enteric Agar (HE) (BD, Franklin Lakes, NJ; cat # 285340)

10 GENERAL OUTLINE OF STUDY

- 1) The cantaloupe was divided into three treatment groups, each weighing approximately 100g.
- 2) The challenge dose of *Salmonella* was applied onto the matrix samples' cut surfaces. *Salmonella* cultures were evenly spread onto all cut sides of the cantaloupe sample surfaces using hockey sticks. One sample was not treated with *Salmonella* cultures as the uncontaminated, untreated control.
- 3) The samples were covered loosely and the *Salmonella* were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
- 4) Water (control) or SalmoFresh™ was applied as described in section 7. Samples in Group A were treated with 1x10⁹ PFU/mL SalmoFresh™ at 4mL / lb, samples in Group B were treated with 1x10⁹ PFU/mL SalmoFresh™ at 2mL / lb, and samples in Group C were treated with water at 4mL / lb. Treatments were evenly applied to the cantaloupe samples cut surfaces.
- 5) The samples were covered and incubated at room temperature for ca. 5 minutes.
- 6) At 5 minutes post-treatment with water or SalmoFresh™, from each sample group, triplicate ~25g samples of cantaloupe were removed, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds.
- 7) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached cantaloupe/peptone water mixture onto separate HE plates supplemented with nalidixic acid (25 µg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\text{Total CFU}}{\text{g of treated cantaloupe}} = \frac{\text{CFU}}{\text{0.5mL plating}} \times \frac{\text{225 mL peptone}}{\text{25 g sample}}$$

Counts from 0.5 mL plating were used during the analysis, because they provided most robust, countable numbers (i.e., more than 10 whenever possible but less than 100 colonies per plate).

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #SF12G16ML1

Group	Weight (g)	<i>Salmonella</i>	Treatment	25g Samples	CFU in 0.5 mL	CFU/g
A (Test)	100	Yes	SalmoFresh 4mL / lb	3	8, 10, 8	144, 180, 144
B (Test)	100	Yes	SalmoFresh 2mL / lb	3	21, 14, 15	378, 252, 270
C (Control)	100	Yes	Water 4mL / lb	3	98, 86, 61	1764, 1548, 1098

11.2 Tabular presentation of results

Table 2 Reduction of *Salmonella* counts on cantaloupe treated with ca. 1×10^9 PFU/mL SalmoFresh when applied at 4mL per lb and 2mL per lb

Group	Challenged with <i>Salmonella</i>	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Significant?
A (Test)	Yes	SalmoFresh 4mL / lb	$n = 3$	156	89%	Yes
B (Test)	Yes	SalmoFresh 2mL / lb	$n = 3$	300	80%	Yes
C (Control)	Yes	Water 4mL / lb	$n = 3$	1470	-	

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

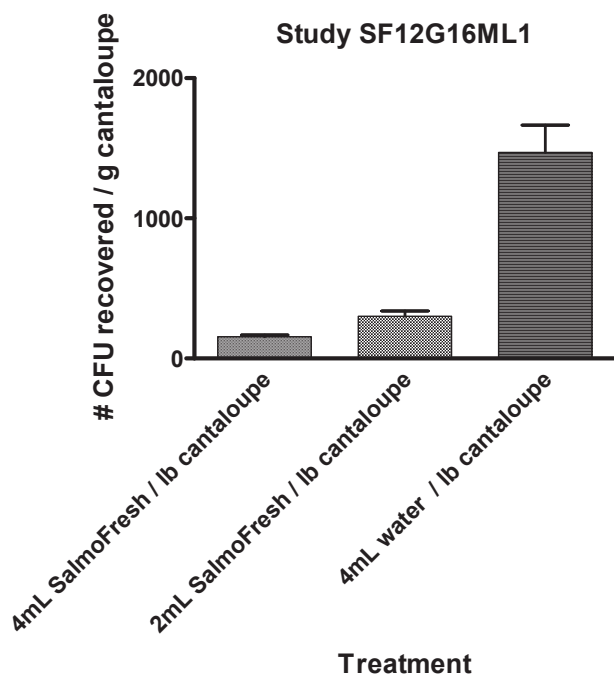
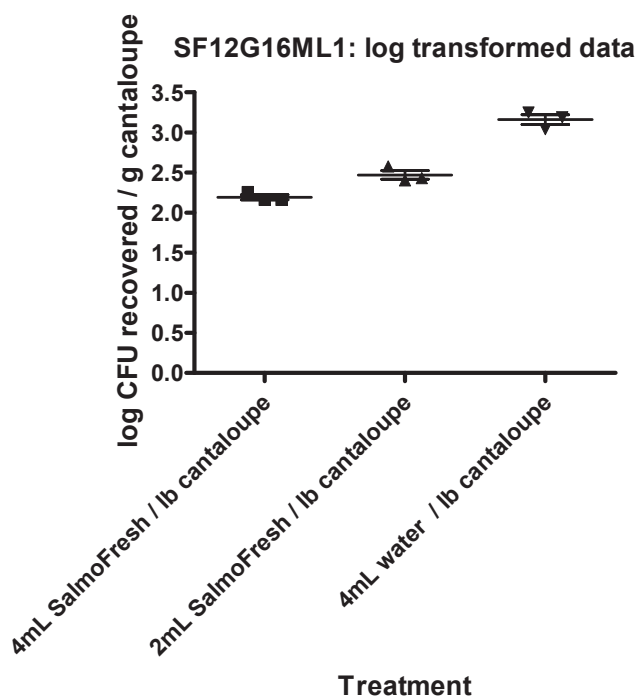


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the SalmoFresh treatment in reducing the number of viable *Salmonella* in the experimentally contaminated cantaloupe was evaluated by comparing the data obtained with the water-treated control samples and the SalmoFresh-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 4.0 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is 0.0004, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
SalmoFresh 4mL / lb vs SalmoFresh 2mL / lb	-144.00	1.244	ns P>0.05
SalmoFresh 4mL / lb vs Water	-1314.0	11.355	*** P<0.001
SalmoFresh 2mL / lb vs Water	-1170.0	10.110	*** P<0.001

11.5 Brief discussion of results and study's conclusions

- Applying ca. 1×10^9 PFU/mL SalmoFresh at 4mL / lb cantaloupe reduced the number of viable *Salmonella* by ca. 89% after 5 minutes of incubation at RT. The observed reduction was statistically significant ($P < 0.001$.)
- Applying ca. 1×10^9 PFU/mL SalmoFresh at 2mL / lb cantaloupe reduced the number of viable *Salmonella* by ca. 80% after 5 minutes of incubation at RT. The observed reduction was statistically significant ($P < 0.001$.)
- The difference in *Salmonella* recovery between samples treated with SalmoFresh at 4mL / lb vs. SalmoFresh at 2mL / lb were not statistically significant.

12 SUMMARY CONCLUSION OF THE STUDY

- SalmoFresh TM can significantly reduce viable *Salmonella* levels in experimentally contaminated cantaloupe by ca. 80-89% in 5 minute contact time, when 1×10^9 PFU/mL SalmoFresh is used at 2mL / lb - 4mL / lb.

- Using a lower SalmoFresh™ application rate (2mL / lb) vs. a higher SalmoFresh™ application rate (4mL / lb) does not significantly affect the efficacy.

13 SIGNATURES

(b) (6)



Manrong Li

Research Scientist

(b) (6)



Alexander Sulakvelidze, Ph.D.

Study Director



**Evaluation of the ability of SalmoFresh to
reduce *Salmonella* contamination in
experimentally contaminated raw tuna**

Study # SF12G16ML2

Intralytix

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1 STUDY TITLE

Evaluation of the ability of SalmoFresh™ to reduce *Salmonella* contamination in experimentally contaminated raw tuna

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Manrong Li, MD	Sr. Research Scientist	Hands-on-research

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
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Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of SalmoFresh™ reduces the number of viable *Salmonella* on raw tuna when applied at the rate of 4mL per lb or 2mL per lb.

6 TEST MATRIX

Raw, sushi-grade tuna was obtained from a local Baltimore supermarket. It was not washed or pre-treated prior to our studies.

7 SALMOFRESH LOT AND APPLICATION RATE

- SalmoFresh™ Lot# 0211C150168
- Titer: approx. 1×10^9 PFU/mL
- SalmoFresh™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).
- The application rate was ca. 4mL SalmoFresh™ per lb or 2mL SalmoFresh™ per lb tuna.

8 SALMONELLA STRAINS USED TO EXPERIMENTALLY CONTAMINATE TUNA

The tuna test matrix was experimentally contaminated with *Salmonella* strains:

- *S.E900*: A nalidixic acid resistant mutant developed from *S.E660* (also known as ATCC13076, *Salmonella enterica* serotype Enteritidis.)
- *S.Ty901*: A nalidixic acid resistant mutant developed from *S.Ty653* (also known as ATCC6539, *Salmonella enterica* serotype Typhi.)
- *S.He902*: A nalidixic acid resistant mutant developed from *S.He899* (also known as ATCC8326, *Salmonella enterica* serotype Heidelberg.)

The strains were selected for nalidixic acid resistance by serially passaging the original isolates on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strains underwent ≤ 8 serial passages before they were determined to be nalidixic acid-resistant at a concentration of 25 $\mu\text{g/mL}$. After the passaging, the above-noted Intralytix strain designations were assigned (i.e., *S.E900*, *S.Ty901*, *S.He902*). The strains were stored at -80°C , at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 μg of nalidixic acid/mL.

Shortly before performing the study, the strains were thawed and grown ($37 \pm 2^\circ\text{C}$, 16-24 h) in LB broth supplemented with nalidixic acid (25 $\mu\text{g/mL}$). Overnight growth corresponds to ca. 4×10^8 CFU/mL. The cultures were mixed in equal parts and the mixture was diluted 1000-fold just prior to performing the study.

The tuna were experimentally contaminated by ca. 2000 CFU of the above-defined 1:1:1 mixture of three *Salmonella* strains / g of tuna.

9 MEDIA AND REAGENTS

- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Hektoen-Enteric Agar (HE) (BD, Franklin Lakes, NJ; cat # 285340)

10 GENERAL OUTLINE OF STUDY

- 1) The tuna was divided into three treatment groups, each weighing approximately 100g.
- 2) The challenge dose of *Salmonella* was applied onto the matrix samples' surfaces. *Salmonella* cultures were evenly spread onto all sides of the tuna sample surfaces using hockey sticks. One sample was not treated with *Salmonella* cultures as the uncontaminated, untreated control.
- 3) The samples were covered loosely and the *Salmonella* were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
- 4) Water (control) or SalmoFresh™ was applied as described in section 7. Samples in Group A were treated with 1x10⁹ PFU/mL SalmoFresh™ at 4mL / lb, samples in Group B were treated with 1x10⁹ PFU/mL SalmoFresh™ at 2mL / lb, and samples in Group C were treated with water at 4mL / lb. Treatments were evenly applied to the tuna samples' surfaces.
- 5) The samples were covered and incubated at room temperature for ca. 5 minutes.
- 6) At 5 minutes post-treatment with water or SalmoFresh™, from each sample group, triplicate ~25g samples of tuna were removed, placed into sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached for a minimum of 30 seconds.
- 7) The number of viable *Salmonella* in the samples was determined by plating aliquots (0.1 mL and 0.5 mL) of the stomached tuna/peptone water mixture onto separate HE plates supplemented with nalidixic acid (25 µg/mL). The plates were incubated (35 ± 2°C, 24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\text{Total CFU}}{\text{g of treated tuna}} = \frac{\text{CFU}}{\text{0.5mL plating}} \times \frac{225 \text{ mL peptone}}{25 \text{ g sample}}$$

Counts from 0.5 mL plating were used during the analysis, because they provided most robust, countable numbers (i.e., more than 10 whenever possible but less than 100 colonies per plate).

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #SF12G16ML2

Group	Weight (g)	<i>Salmonella</i>	Treatment	25g Samples	CFU in 0.5 mL	CFU/g
A (Test)	100	Yes	SalmoFresh 4mL / lb	3	6, 5, 9	108, 90, 162
B (Test)	100	Yes	SalmoFresh 2mL / lb	3	14, 17, 20	252, 306, 360
C (Control)	100	Yes	Water 4mL / lb	3	86, 71, 99	1548, 1278, 1782

11.2 Tabular presentation of results

Table 2 Reduction of *Salmonella* counts on tuna treated with ca. 1×10^9 PFU/mL SalmoFresh when applied at 4mL per lb and 2mL per lb.

Group	Challenged with <i>Salmonella</i>	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Significant?
A (Test)	Yes	SalmoFresh 4mL / lb	$n = 3$	120	92%	Yes
B (Test)	Yes	SalmoFresh 2mL / lb	$n = 3$	306	80%	Yes
C (Control)	Yes	Water 4mL / lb	$n = 3$	1536	-	

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

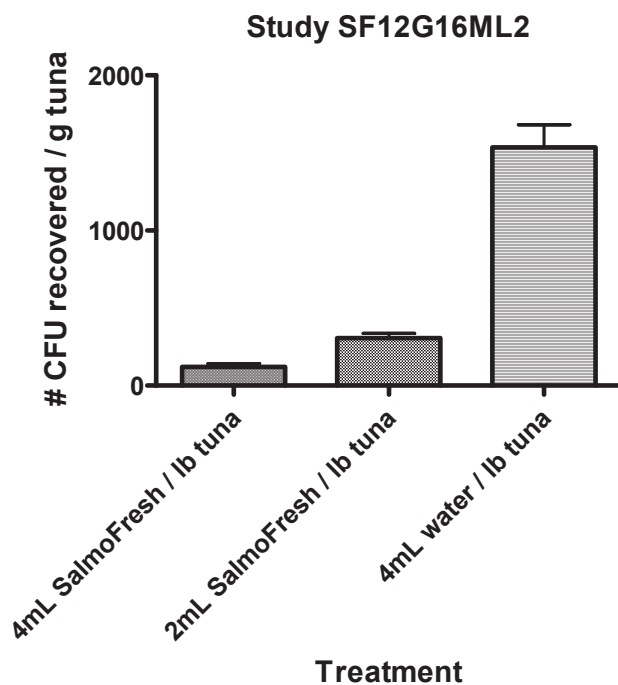
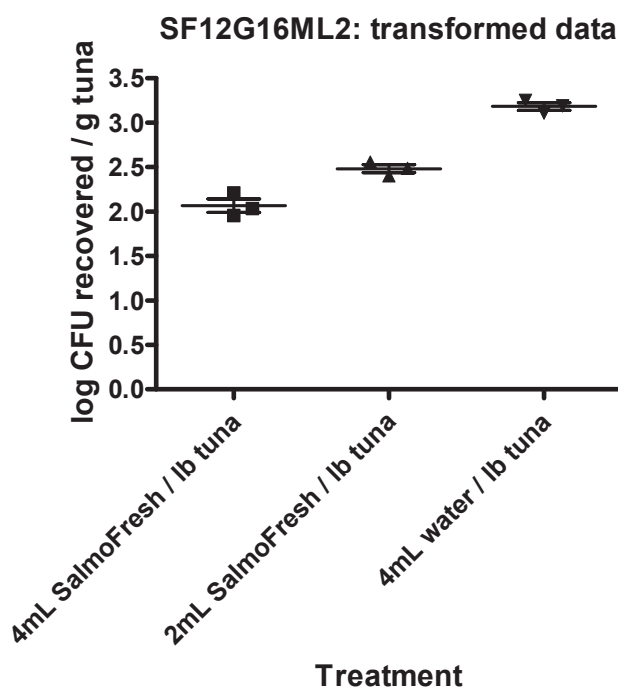


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the SalmoFresh treatment in reducing the number of viable *Salmonella* in the experimentally contaminated raw tuna was evaluated by comparing the data obtained with the water-treated control samples and the SalmoFresh-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 4.0 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

One-way Analysis of Variance (ANOVA)

The P value is <0.0001, considered extremely significant. Variation among column means is significantly greater than expected by chance.

Comparison	Mean Difference	q	P value
SalmoFresh 4mL / lb vs SalmoFresh 2mL / lb	-186.00	2.141	ns P>0.05
SalmoFresh 4mL / lb vs Water	-1416.0	16.298	*** P<0.001
SalmoFresh 2mL / lb vs Water	-1230.0	14.158	*** P<0.001

11.5 Brief discussion of results and study's conclusions

- Applying ca. 1×10^9 PFU/mL SalmoFresh at 4mL / lb tuna reduced the number of viable *Salmonella* by ca. 92% after 5 minutes of incubation at RT. The observed reduction was statistically significant ($P < 0.001$.)
- Applying ca. 1×10^9 PFU/mL SalmoFresh at 2mL / lb tuna reduced the number of viable *Salmonella* by ca. 80% after 5 minutes of incubation at RT. The observed reduction was statistically significant ($P < 0.001$.)
- The difference in *Salmonella* recovery between samples treated with SalmoFresh at 4mL / lb vs. SalmoFresh at 2mL / lb were not statistically significant.

12 SUMMARY CONCLUSION OF THE STUDY

- SalmoFresh™ can significantly reduce viable *Salmonella* levels in experimentally contaminated raw tuna by ca. 80-92% in 5 minute contact time, when 1×10^9 PFU/mL SalmoFresh is used at 2mL / lb - 4mL / lb.

- Using a lower SalmoFresh™ application rate (4mL / lb) vs. a higher SalmoFresh™ application rate (2mL / lb) does not significantly affect the efficacy.

13 SIGNATURES

(b) (6)



Manrong Li

Research Scientist

(b) (6)



Alexander Sulakvelidze, Ph.D.

Study Director



**Evaluation of the ability of SalmoFresh™ to
reduce *Salmonella* contamination in
experimentally contaminated romaine lettuce**

Study # SF12H16AP1

Intralytix

The Columbus Center

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1 STUDY TITLE

Evaluation of the ability of SalmoFresh™ to reduce *Salmonella* contamination in experimentally contaminated romaine lettuce

2 STUDY DIRECTOR

Alexander Sulakvelidze, Ph.D.

3 STUDY PERSONNEL

Name:	Title:	Role:
Alexander Sulakvelidze, Ph.D.	Chief Scientist	Study Director
Adam Parks, Ph. D.	Senior Research Scientist	Hands-on-research

4 PERFORMING LABORATORY

Intralytix, Inc.
Research and Development
The Columbus Center
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Baltimore, MD 21202

5 STUDY OBJECTIVE

To determine whether application of SalmoFresh™ reduces the number of viable *Salmonella* on romaine lettuce with application of 13.6ml SalmoFresh™ per pound of lettuce.

6 TEST MATRIX

Organic romaine lettuce was obtained from a Frederick, MD supermarket. It was not washed or pre-treated prior to our studies.

7 SALMOFRESH™ LOT AND APPLICATION RATE

- SalmoFresh™ Lot# 0211K110119
- Titer: approx. 1×10^9 PFU/mL
- SalmoFresh™ was applied using Basic Spray Gun Model #250 (Badger Air-Brush Co., Franklin Park, IL).
- The application volume was ca. 13.6ml SalmoFresh™ per pound of romaine lettuce.

8 SALMONELLA STRAINS USED TO EXPERIMENTALLY CONTAMINATE ROMAINE LETTUCE

The romaine lettuce test matrix was experimentally contaminated with *Salmonella* strains:

- *S.E900*: A nalidixic acid resistant mutant developed from *S.E660* (also known as ATCC13076, *Salmonella enterica* serotype Enteritidis.)
- *S.Ty901*: A nalidixic acid resistant mutant developed from *S.Ty653* (also known as ATCC6539, *Salmonella enterica* serotype Typhi.)
- *S.He902*: A nalidixic acid resistant mutant developed from *S.He899* (also known as ATCC8326, *Salmonella enterica* serotype Heidelberg.)

The strains were selected for nalidixic acid resistance by serially passaging the original isolates on LB agar plates supplemented with increasing concentrations of nalidixic acid. The strains underwent ≤ 8 serial passages before they were determined to be nalidixic acid-resistant at a concentration of 25 $\mu\text{g/ml}$. After the passaging, the above-noted Intralytix strain designations were assigned (i.e., *S.E900*, *S.Ty901*, *S.He902*). The strains were stored at -80°C , at Intralytix, in 70% LB broth/30% glycerol supplemented with 25 μg of nalidixic acid/ml.

Shortly before performing the study, the strains were streak purified from frozen stocks on LB plates. Representative colonies were grown ($37 \pm 2^\circ\text{C}$, 16-24 h) in NZCYM broth supplemented with nalidixic acid (25 $\mu\text{g/ml}$.) Overnight cultures were diluted to $\text{OD}_{600} = 1.0$ in NZCYM, corresponding to ca. 6×10^8 CFU/mL. The cultures were mixed in equal parts and the mixture was diluted 10,000-fold just prior to performing the study.

The lettuce was experimentally contaminated by ca. 3000 CFU of the above-defined 1:1:1 mixture of three *Salmonella* strains / g of lettuce.

9 MEDIA AND REAGENTS

- NZCYM (Becton, Dickinson and Co., Sparks, MD; cat #240410)
- LB (Neogen, Lansing, MI; catalog # 7279)
- Nalidixic acid (Acros Organics, Fair Lawn, NJ; catalog # AC16990-1000)
- Peptone water (Becton, Dickinson and Co., Sparks, MD; cat #218105)
- Hektoen-Enteric Agar (HE) (BD, Franklin Lakes, NJ; cat # 285340)

10 GENERAL OUTLINE OF STUDY

- 1) The lettuce was divided into two treatment groups with two replicates each, each weighing approximately 15g.
- 2) The challenge dose of *Salmonella* was applied onto the matrix samples' surfaces. *Salmonella* cultures were evenly spread onto the lettuce surfaces using hockey sticks. One sample was not treated with *Salmonella* cultures as the uncontaminated, untreated control.
- 3) The samples were covered loosely and the *Salmonella* were allowed to colonize the matrix samples' surfaces at room temperature (RT) for 60 min.
- 4) Phosphate buffered saline (PBS, control) or SalmoFresh™ was applied as described in section 7. Samples in Group A were treated with 1×10^9 PFU/mL SalmoFresh™ at 13.6ml per pound of lettuce, samples in Group B were treated with phosphate buffered saline at 13.6ml per pound of lettuce. Treatments were evenly applied to the lettuce samples surfaces using the spray gun.
- 5) The samples were covered and incubated at room temperature for ca. 5 minutes.
- 6) At 5 minutes post-treatment with PBS or SalmoFresh™, the duplicate samples of lettuce were placed into separate sterile bags, and 225 mL of sterile peptone water was added. The bags were hand mushed briefly and stomached at 230 rpm for a minimum of 30 seconds.
- 7) The number of viable *Salmonella* in the samples was determined by plating in triplicate 0.1 ml of the stomached lettuce/peptone water mixture onto separate HE plates supplemented with nalidixic acid (25 µg/mL). The plates were incubated ($35 \pm 2^\circ\text{C}$,

24±2 hr), and the CFU/g of sample were calculated after counting the colonies, as follows:

$$\frac{\text{Total CFU}}{\text{g of treated lettuce}} = \frac{\text{CFU}}{\text{0.1mL plating}} \times \frac{\text{225 mL peptone}}{\text{g sample}}$$

11 RESULTS

11.1 Raw Data

Table 1 Raw Data for Study #SF12H16AP1

Group	Weight (g)	<i>Salmonella</i>	Treatment	~15g Samples	CFU in 0.1 mL	CFU/g
A (Test)	15.5, 16.7	Yes	SalmoFresh™ 13.6ml / pound of lettuce	2	15, 19, 13, 22, 37, 28	1, 1, 1, 2, 2, 1
B (Control)	12.4, 8.9	Yes	PBS 13.6ml / pound of lettuce	2	143, 210, 155, 178, 227, 186	14, 18, 15, 16, 24, 17, 17

11.2 Tabular presentation of results

Table 2 Reduction of *Salmonella* counts on lettuce treated with ca. 1 x10⁹ PFU/mL SalmoFresh™ when applied at 13.6 ml per pound.

Group	Challenged with <i>Salmonella</i>	Treatment	Replicates	Mean CFU/g	Percent reduction vs. water	Significant?
A (Test)	Yes	SalmoFresh™ 13.6ml / pound of lettuce	<i>n</i> =6	1.4	92%	Yes (<i>P</i> <.0001)
B (Control)	Yes	PBS 13.6ml / pound of lettuce	<i>n</i> = 6	17.2	-	

11.3 Graphical presentation of results

Chart constructed using raw data (mean with SEM)

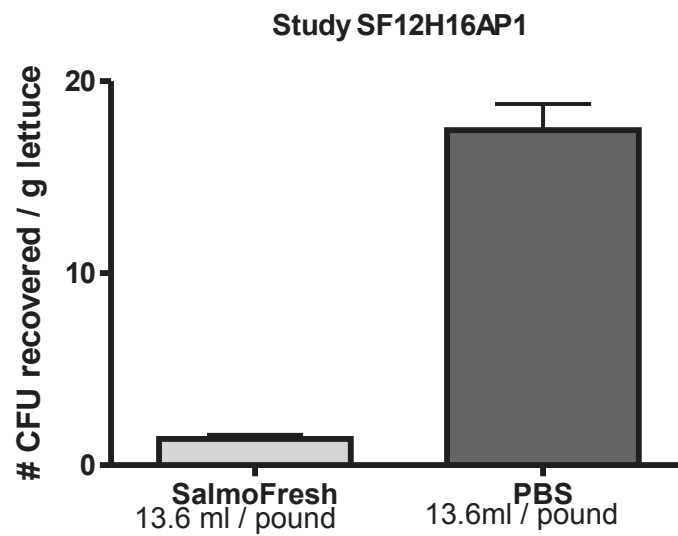
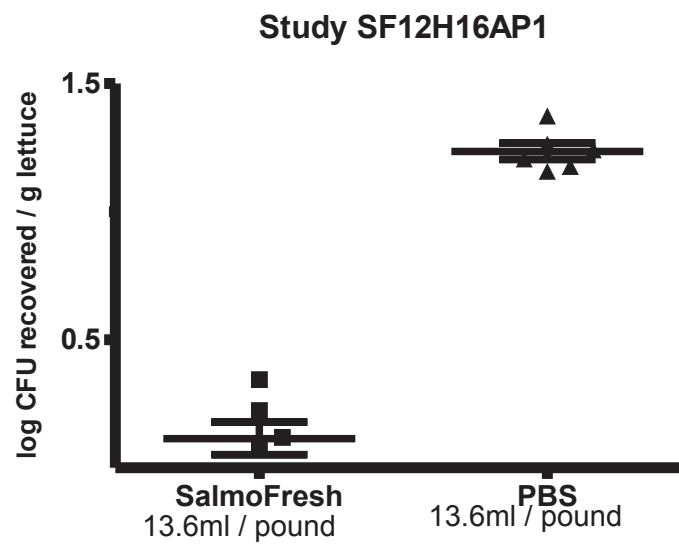


Chart constructed using log-transformed data



11.4 Statistical analysis

The efficacy of the SalmoFresh™ treatment in reducing the number of viable *Salmonella* in the experimentally contaminated lettuce was evaluated by comparing the data obtained with the PBS-treated control samples and the SalmoFresh™-treated samples.

Statistical analysis was performed using version 3.05 of GraphPad InStat and version 4.0 of GraphPad Prism (GraphPad Software, San Diego, CA; www.graphpad.com)

Unpaired T-Test with Welch correction

SalmoFresh™ 13.6 ml / pound vs PBS 13.6 ml / pound	*** $P < 0.0001$
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The P value is < 0.0001 , considered extremely significant.

11.5 Brief discussion of results and study's conclusions

- Applying ca. 1×10^9 PFU/mL SalmoFresh™ at 13.6ml per pound romaine lettuce reduced the number of viable *Salmonella* by ca. 92% after 5 minutes of incubation at RT. The observed reduction was statistically significant ($P < 0.001$.)

12 SUMMARY CONCLUSION OF THE STUDY

- SalmoFresh™ can significantly reduce viable *Salmonella* levels in experimentally contaminated lettuce by ca. 92% in 5 minute contact time, when 1×10^9 PFU/mL SalmoFresh™ is used at 13.6ml per pound.

13 SIGNATURES

(b) (6)



Adam Parks

Senior Research Scientist

(b) (6)



Alexander Sulakvelidze, Ph.D.

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